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Metabolic study of Alzheimer's disease using mutant *C. elegans*

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Abstract

Alzheimer's disease (AD) is associated with chronic neurodegeneration and is the cause of the most common form of dementia. The main hallmarks of AD are aggregates of amyloid beta (Aβ) and formation of hyperphosphorylated tau neurofibrillary tangles (NFTs).

Unfortunately, after more than 100 years of research the exact cause(s) and mechanisms involved in AD progression remain unclarified. Evidence indicates that impaired mitochondrial bioenergetics may precede by decades, the neurodegeneration and cognitive decline associated with AD. Moreover, a genetic association of the core metabolic enzyme dihydrolipoamide dehydrogenase (DLD) with late-onset AD and reduced activity of DLD-containing enzymes suggests glucose hypo-metabolism as a possible cause of AD. Contrary to this, improvements of AD symptoms under caloric restriction or other means of reducing-glucose dependent energy metabolism supports an alternative hypothesis that a decrease in glucose metabolism may be protective. In the present study, we used mutant *Caenorhabditis elegans* (*C. elegans*) to investigate the effect of glucose metabolism on AD progression. We initially looked at the role of suppressed DLD-1, and then we focused on the effect of high glucose in AD pathogenesis.

Transgenic expression of Aβ in *C. elegans* causes both phenotypic and behavioral defects and results in accumulation of toxic Aβ oligomers, culminating in protein aggregation as is normally associated with AD pathophysiology. Aβ expression in worm muscle causes age-dependent progressive paralysis and impaired acetylcholine neurotransmission while neuronal Aβ expression results in defective chemotaxis and impaired serotonin sensitivity as well as reduced fecundity and egg hatching. Suppression of the *dld-1* gene alleviated paralysis, improved acetylcholine neurotransmission, enhanced chemotaxis and restored normal sensitivity to serotonin. *dld-1* gene suppression also protects vitality as indicated by improved fecundity and egg hatching. Interestingly, protective effects of *dld-1* suppression could be reversed using the calcium ionophore (CaI), indicating that the protective mechanism involves calcium signaling. Each of these beneficial effects of *dld-1* gene suppression could be mimicked using a specific, small molecule inhibitor of the DLD-1 enzyme, 5-methoxyindole-2-carboxylic acid (MICA).

High glucose levels are associated with the metabolic disorder, diabetes, which is a major risk factor for AD. In fact, it has been suggested that AD is a neural form of diabetes, type 3 diabetes. If true, drugs used to treat diabetes could act as possible treatments for AD. In this study, I investigated the effect of elevated glucose, the glucose metabolism inhibitor, 2-deoxy-d-glucose

(2DOG) as well as the anti-diabetes drugs, metformin and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), on AD progression. Elevated glucose in the growth medium induced hyperactivity, which interfered with the paralysis assays, but it was seen to impair cholinergic neurotransmission, egg laying and hatching. In contrast, 2DOG, metformin and AICAR each significantly alleviated the behavioural problems associated with Aβ-expression. These results suggest that regulating glucose metabolism and anti-diabetes drugs may be effective in treating AD.

Time-dependent accumulation of Aβ worsens behavioral defects in AD. Recent strategies to overcome AD pathogenesis have targeted Aβ oligomerization. Suppression of the *dld-1* gene, as well as treatment with MICA, 2DOG, metformin and AICAR, each decreased the formation of toxic Aβ oligomers, whereas glucose enrichment enhanced Aβ oligomerization dose-dependently.

An alternative strategy to treat AD is to decrease phosphorylation of tau, as hyperphosphorylation leads to the formation of NFTs. Experimental induction of *O*-GlcNAcylation, either by suppressing the *O*-GlcNAcase enzyme with Thiamet-G (TMG) or by suppressing the *O*-GlcNAcase gene (*oga-1*) itself, resulted in the expected decrease in tau phosphorylation. Furthermore, suppression of the O-GlcNAc transferase gene (*ogt-1*), which is necessary for *O*-GlcNAcylation, caused an increase in tau phosphorylation. Elevated dietary glucose was expected to induce *O*-GlcNAcylation, leading to the inhibition of tau phosphorylation. Instead, glucose significantly induced tau phosphorylation at critical residues. Moreover, treatment with TMG could not prevent the glucose-mediated increase in tau phosphorylation. Thus, glucose is a risk factor in tau phosphorylation that is independent of GlcNAcylation of tau in this *C. elegans* model of AD. While this work suggests that enhancing *O*-GlcNAcylation to prevent tau phosphorylation may be beneficial, care must be taken to avoid elevated glucose levels.

Interestingly, both TMG and *oga-1* suppression caused a decrease in *Aβ*-mediated symptoms, whereas suppression of *ogt-1* had the opposite effect. This strongly implicates *O*-GlcNAcylation in the toxicity of the *Aβ* peptide despite no previously evidence to suggest this.

In conclusion, our results highlight the importance of active energy metabolism on AD progression. Suppression of the *dld-1* gene, a key factor in energy metabolism, exposure to the glucose analogue 2DOG, and treatment with the diabetes drug, metformin, all protected against Aβ- and tau-mediated toxicity in the *C. elegans* models.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed papers

1- W. Ahmad, Overlapped metabolic and therapeutic links between Alzheimer and diabetes, Molecular neurobiology, 47 (2013) 399-424 [1].

2- W. Ahmad, P.R. Ebert, Metformin Attenuates Abeta Pathology Mediated Through Levamisole Sensitive Nicotinic Acetylcholine Receptors in a *C. elegans* Model of Alzheimer's Disease, Molecular neurobiology, DOI 10.1007/s12035-016-0085-y(2016) [2].

Conference abstracts

1. *dld-1* suppression attenuates the detrimental effects of amyloid beta deposition in a *Caenorhabditis elegans* model of Alzheimer's Disease. 3rd Molecular Neurodegeneration Conference, Cannes, France (10-12 Sep, 2013)

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1- **Overlapped metabolic and therapeutic links between Alzheimer and diabetes, Molecular neurobiology, 47 (2013) 399-424 [1] – incorporated as Chapter II**

Contributor	Statement of contribution
Waqar Ahmad (Candidate)	Wrote the paper (100%)

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Waqar Ahmad (Candidate)	Wrote the paper (100%)
Paul R. Ebert	Edited paper (100%)

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Keywords

Alzheimer's disease, glucose energy metabolism, dihydrolipoamide dehydrogenase, beta-amyloid, tau, oligomerization, phosphorylation, O-GlcNAcylation, neurotoxicity, phosphine

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Fields of Research (FoR) Classification

0304 Medicinal and Biomolecular Chemistry – 25%

0601 Biochemistry and Cell Biology – 25%

1109 Neurosciences – 50%

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List of Abbreviations used in the thesis

2-deoxy-d-glucose	2DOG
2-methoxyindole-5-carboxylic acid	MICA
5-aminoimidazole-4-carboxamide ribonucleotide	AICAR
3'-phosphoinositide-dependent-kinase-1	PDK-1
5-hydroxycystosine	5-OHC
5-hydroxyuracil	5-OHU
8-hydroxyadenine	8-OHA
8-hydroxyguanine	8-OHG
Advanced glycation end products	AGEs
Aldehydes	HCOR
Alkoxyl	$RO^{\cdot}$
Alzheimer's disease	AD
Amyloid beta	Aβ
Arachidonic acid	AA
Arginine	Arg
Blood brain barrier	BBB
Branched chain ketoacid dehydrogenase	BCKDH
Bristol isolate of *C. elegans*	N2
Caenorhabditis elegans	*C. elegans*
Calcium ionophore	CaI
Caloric restriction	CR
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone	FCCP
Cerebral metabolic rate of glucose	CMRglc
Cerebrospinal fluid	CSF
Chemotaxis index	CI
Cholesterol acyltransferase 1	ACAT1
Cyclooxygenase	COX
Dietary restriction	DR
Dihydrolipoamide dehydrogenase	DLD/ DLD-1/ *dld-1*
Docosahexaenoic	DHA
Eicosapentaenoic acid	EPA
Free fatty acids	FFA
Glucose transporters	GLUTs
Glycine cleavage system	GCS
Glycogen synthase kinase 3	GSK3
High density lipoproteins	HDL
Histidine	His
Hydrochlorous acid	HOCL
Hydrogen peroxide	H_2O_2
Hydroperoxyl	$HO_2^{\cdot}$
Hydroxyl	$OH^{\cdot}$
Insulin degrading enzyme	IDE
Insulin receptor substrates	IRS
Insulin resistance	IR
Islet amyloid polypeptide	hIAPP/ amylin
Long-term potentiating	LPT
Low density lipoproteins	LDL
Mammalian target of rapamycin	mTOR
Mild cognitive impaired	CMI

Mitogen-activated protein kinases	MAPK
Nematode growth medium	NGM
Neurofibrillary tangles	NFTs
Nicotinamide adenine dinucleotide	NAD
Nitric oxide	$NO^{\cdot}$
O-GlcNAcase	OGA/ oga-1
Organic peroxides	ROOH
Oxysterol 24S-hydroxycholesterol	24OHC
O-β-GlcNAcylation	O-GlcNAc
Parts per million	ppm
Peroxyl	$ROO^{\cdot}$
Peroxynitrite	$ONOOH/ ONOO^{-}$
Phophoinositide phosphates	PIP3
Phospholipase isoform A2	PLA2
Positron emission tomography	PET
Post-transcriptional gene silencing	PTGS
Posttranslational modifications	PTMs
Proliferator-activated receptor-γ	PPARγ
Proline	Pro
Protein kinase C	PKC
Pyruvate dehydrogenase	PDH
Reactive oxygen species	ROS
Receptors for advanced glycation end products	RAGEs
Retinoid X receptors	RXRs
Serine	Ser
streptozotocin	STZ
Superoxide dismutases	SODs
Thiamet-G	TMG
Threonine	Thr
Time for ¾ of the worms to die	LT75
Time for half of the worms to die	LT50
Tumour necrosis factor	TNF
Type 2 diabetes mellitus	T2DM
Uncoupling proteins	UCPs
α-Ketoglutarate dehydrogenase	KGDH
α-linoleic acid	ALA

CHAPTER # I: General Introduction

Alzheimer's disease (AD) is associated with chronic neurodegeneration and is the cause of the most common form of dementia [3]. Currently, more than 46 million people are suffering from AD, and this number is expected to reach more than 100 million by the end of 2050 if effective treatments for AD are not discovered [4]. The “Alzheimer’s disease” was named on the German clinical psychiatrist and neuro-chemist Alois Alzheimer. He started a long- term study of a female patient showing the signs of memory disturbance, confusion, paranoia and aggression in 1901. After her death in 1906, Alzheimer reported the presence of plaques and neurofibrillary tangles (NFTs) in the post-mortem brain. The structure and composition of both plaques and NFTs were determined years after this discovery. The term amyloid was introduced by Rudolph Virchow in 1854 after staining the brain tissue with iodine [5]. Aβ was first isolated and sequenced by Glenner and Wong as a lower molecular weight polypeptide and reported as a major component of vascular plaques in 1984 [6], while in 1986, tau was identified as major component on NFTs [7, 8]. Till date production and accumulation of both amyloid plaques and NFTs are considered as prime reasons for AD.

There are two primary schools of thought regarding the cause of AD, the first of which is that production and deposition of Aβ from abnormally cleavage amyloid beta precursor protein (APP) is primarily responsible (the Aβ cascade). APP is a type-1 transmembrane protein and has been suggested to play a role in synaptogenesis, neuronal protein trafficking, calcium metabolism, cell adhesion, and transmembrane signal transduction. During normal functioning, APP proteolyzed into various fragments. APP cleavage by α-secretase resulted in the formation of large and soluble ectodomain of APP (often called as sAPPα). This cleavage occurs within Aβ region thus protects formation of Aβ. Aβ is 39-42 residues peptide and generated after sequential cleavages of APP by β-secretase followed by γ-secretase complexes [9-11]. Excessive production and defective Aβ clearance leads to AD by blocking neurotransmission, reducing neuronal synapsis, inducing oxidative stress and promoting mitochondrial dysfunction. Aβ can occur in monomeric or oligomeric form [12]. Aβ monomers are considered non-toxic to neurons whereas small soluble Aβ oligomers are likely to be responsible for Aβ-mediated neurotoxicity [13]. This is presumed because an early cognitive decline appears before the deposition of oligomers to form plaques in patients with AD [14-20]. Furthermore, drug screening studies demonstrate that reduction in Aβ oligomers is beneficial even if the level of monomers increase highlighting the key role of Aβ oligomers in disease progression [21-24].

A second school of thought is that formation of NFTs initiates synaptic loss and neural degeneration in Alzheimer's disease. The NFTs are caused by disruption of microtubules assembly due to hyperphosphorylation of tau, leading to deranged neuronal cell body structure and the symptoms of AD [25, 26].

There is also a tertiary school of thought that is much less prominent than the other two. The third proposal is that the pathogenicity of Alzheimer's disease is associated with impaired central energy metabolism. A decline in both glucose metabolism and ATP formation caused by a decrease in the level of metabolites and enzyme activity of glycolysis and the TCA cycle was observed in the brains of AD patients [27, 28]. This decrease in energy metabolism was interpreted in two contrasting hypotheses i) as a main cause of AD, or ii) as a protective measure against the disease pathogenesis. Unfortunately, in AD; it is difficult to distinguish cause from the consequence due to unavailability of AD brain samples during disease progression. How glucose energy metabolism regulates AD progression is still under investigation. It is well known that both Aβ and NFTs impair mitochondrial functions [29, 30]. Suppression of glucose energy metabolism (oxidative phosphorylation) showed reduction in Aβ toxicity in model systems as well as in neuronal cell lines [31, 32] whereas, increase in oxidative phosphorylation was found to enhance the Aβ toxicity [33]. These observations suggest a possible role of energy metabolism in AD progression where suppression in glucose metabolism was found to reduce Aβ toxicity.

Increased risk of late-onset Alzheimer's disease is genetically associated with four single nucleotide variants of *dld*, though how each variant specifically affects DLD enzyme activity is still unknown [34]. The *dld* gene encodes a core enzyme of energy metabolism in TCA cycle. The DLD enzyme contributes to four major enzyme complexes, pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGDH), branched chain ketoacid dehydrogenase (BCKDH) and the glycine cleavage system (GCS), the first three of which are key contributors to energy metabolism [35]. Several lines of evidence indicate that these dehydrogenase complexes are involved in neurodegenerative disorders. Reduced activities of PDHC and KGDHC have been observed in post-mortem brain tissues and fibroblasts of patients with Parkinson's and Alzheimer's disease [36-40]. These reductions in dehydrogenase complex activity may induce the reduction in glucose utilization as observed in neurodegenerative diseases [41, 42]. Although mice with *dld* gene deletion do not show any deleterious behavioral phenotypes, an increased susceptibility to mitochondrial toxin's modelling neurodegenerative diseases has been observed and attributed to *dld* depletion [43]. However, in healthy rats that do not succumb to neurodegeneration, there is no age-related change in expression of *dld* observed throughout their lifetime [44]. Although these findings suggest a

possible role between disease progression and dehydrogenase complex activities, we cannot conclude a negative role of DLD containing complexes in accelerating progression of AD or other neurodegenerative diseases. It is possible that the decrease in their activities may, in fact, be a protective effect, induced in diseased brains in an attempt to limit disease progression [45]. The recent demonstration that reduction in *dld-1* expression results in an extended lifespan in *C. elegans* suggests a relationship between the effect of energy metabolism on ageing and its effect on ageing-associated diseases, including Alzheimer's disease (AD) [46].

Increased risk of AD in the presence of metabolic disorder disease "diabetes" also highlights the possible role of impaired energy metabolism and AD progression. Diabetes is considered as a prime risk factor for AD and both diseases share many features, such as misfolding of proteins; impaired insulin signaling, a decrease in glucose and lipid metabolism, and mitochondrial dysfunction [1, 47]. It has been suggested that AD is a third type of diabetes.

Current drugs, or those being developed as AD therapeutics, rely on three main strategies to enhance depressed neuronal function; stimulation of neuronal receptors, prevention or clearing of plaques, prevention or elimination of NFTs [48]. Unfortunately, none of the existing drugs for the treatment of AD prevent or reverse the disease, rather they are only used to manage the symptoms. Furthermore, drugs that target Aβ and tau not only have a poor track record, but candidates who address these targets are still in the early development stage and will require a long time for approval. As several studies observe similar pathways involved in both AD and type 2 diabetes mellitus (T2DM), one possibility is that drugs used for T2DM treatment may prevent AD or at least alleviate symptoms [1, 49-52].

Recent attempts to develop therapies against AD based on preventing or clearing misfolded proteins from the brain have all resulted in failure, prompting us to investigate the possibility of a metabolic basis of AD. The nematode, *C. elegans*, is a well-developed genetic model system that has been used extensively to study aging, as well as processes associated with ageing and AD, including energy metabolism and protein misfolding [53, 54]. Specific *C. elegans* models have been developed to study AD in which human Aβ or tau is expressed in either muscles or in neurons. Expression and successive aggregation of Aβ in the muscles leads to progressive paralysis, which provides a convenient phenotype to study molecular processes linked to Aβ toxicity [55]. The *C. elegans* model expressing Aβ in neuronal cells provides behaviour phenotypes for investigation [55-57]. Meanwhile tau expressing *C. elegans* models are capable of highly phosphorylating human tau to an AD-like state, resulting in defects in motor neurons [58]. While expression of Aβ or tau in muscle has been shown to be a useful model, neuronal expression reflects more closely the situation

in human AD and allows study of behaviour phenotypes as well as protein modifications. Genetic or chemical studies are equally convenient in the worm model.

In this study, we are interested to identify a potential association between altered energy metabolism and AD, and how this affects overall pathogenesis. To understand this possible link, we suppressed the *dld-1* gene using RNAi in *C. elegans*. We have also suppressed DLD-1 enzyme using a chemical inhibitor. Meanwhile, suppression of *dld-1* could result in the reduced energy metabolism, we as well examine the effect of glucose enrichment Aβ or tau- mediated toxicity in *C. elegans*. As any impairment in energy metabolism could lead to metabolic disorders, we also investigated the effect of popular anti-diabetic drug metformin in Aβ models of *C. elegans*. This thesis consists of following chapters,

CHAPTER I: General introduction

CHAPTER II: This chapter was published as “Overlapped metabolic and therapeutic links between Alzheimer and diabetes." It serves as literature review to support the current study. In this chapter, I reviewed the common pathways associated with both Alzheimer’s and diabetes especially focusing on energy metabolism.

CHAPTER III: This chapter overviews the techniques used in this study and named as “materials and methods”.

CHAPTER IV: In this chapter, we have investigated the relationship between DLD-1 and Aβ-mediated toxicity in *C. elegans* model system. We found that a decrease in glucose energy metabolism due to *dld-1* suppression was associated with reduced Aβ oligomerization and correspondingly reduced Aβ-mediated symptoms.

CHAPTER V: We explored the effects of *dld-1* suppression by using specific RNAi against Aβ-toxicity. In this study, a *C. elegans* model of AD was exposed to a chemical inhibitor of DLD enzyme known as 2-methoxyindole-5-carboxylic acid (MICA). MICA mimics the effect of *dld-1* suppression through RNAi against Aβ-mediated toxicity in *C. elegans*. Moreover, DLD-1 suppression either by RNAi or chemically was associated with calcium homeostasis as the effects of DLD-1 inhibition can be reversed by the calcium ionophore.

CHAPTER VI: As DLD-1 suppression reduces the rate of glucose energy metabolism, we also investigated the effect of high glucose on AB and tau-mediated toxicity using *C. elegans*. High glucose concentrations not only induce Aβ oligomerization but also increased the phosphorylation

of serine and threonine residues of tau that are considered critical to AD pathogenesis. This phenomenon appears unrelated to glucose-mediated O-GlcNAcylation.

CHAPTER VII: This chapter has been published as “Metformin attenuates Aβ pathology mediated through levamisole sensitive nicotinic acetylcholine receptors in a *C. elegans* model of Alzheimer’s Disease”. Worms fed with high glucose could act as possible models for diabetes. In this chapter, we studied the effect of widely used anti-diabetic drug metformin and tested as possible therapy to counteract Aβ toxicity in *C. elegans*.

CHAPTER VIII: General discussion

CHAPTER # II: Overlapped metabolic and therapeutic links between Alzheimer and diabetes

This chapter has been published as" Ahmad W. Overlapped metabolic and therapeutic links between Alzheimer and diabetes. Mol Neurobiol. 2013 Feb;47(1):399-424. doi: 10.1007/s12035-012-8352-z**"**

Abstract

Alzheimer's disease (AD) and diabetes are among the most common diseases associated with ageing. The pathology of AD is strongly associated with accumulated misfolding proteins that results in neuronal dysfunction within the brain. Diabetes, on the contrary, is characterized by altered insulin signaling that results in reduced glucose uptake, metabolic suppression of energy consuming cells and conversion of glucose to fat in the liver. Despite distinguishing features, these diseases share common elements and may in fact be viewed as fundamentally similar disorders that differ in magnitude of specific traits, primarily affected tissues and time of onset. In this review, we outline the fundamental basis of each of the two diseases and highlight similarities in their pathophysiology. Further ahead we will discuss these features in relation to the development of drugs to treat these two diseases, particularly AD, for which the development of therapeutic chemicals has proven to be particularly difficult. We conclude with comments on efforts to develop a simple organism, *C. elegans*, as a genetic model to be used to study the systems biology of diabetes and AD.

Introduction

AD and diabetes are both age-associated diseases. AD is the most common form of dementia in elderly and is clinically categorized by a progressive loss of cognitive and memory functions. Post-mortem examination of brains of AD sufferers reveals accumulation of extracellular plaques of Aβ peptides and intracellular NFTs consisting of hyperphosphorylated tau protein. In addition, there is a decrease in synapse density and a corresponding decrease in brain volume due to neurodegeneration [59-61]. On the other hand, diabetes mellitus is associated with decreased body insulin production than required (Type 1) and impaired insulin signaling (Type 2/ T2DM). Another hallmark of diabetes is the formation of the Human islet amyloid polypeptide (hIAPP, amylin) that leads to pancreatic β-cells dysfunction. The resulting metabolic disturbance leads to chronic hyperglycemia, which is the immediate cause of many of the symptoms of diabetes [62-64].

Even though the pathologies of these two diseases appear superficially unrelated, diabetes sufferers have an increased risk of development of age-associated neurodegenerative diseases, including AD. In 2005, Suzanne de La Monte introduced the term "type 3 diabetes" about AD. Her team observed insulin impairment associated with AD patient during post-mortem. They observed an inverse correlation between insulin receptor abundance and the Braak score of AD brains (Braak method scores NFTs levels within transentorhinal region within the brain. If the NFTs are confined to transentorhinal region, it will be called as stage, I or II depending on their concentration. If the NFTs spread to limbic regions, the stage III or IV will appear. Stage V and VI appear when NFTs are also found in neocortical environment), with 80% reduced IRS levels in the most extreme cases. They observed decreased mRNA levels of IGF-1 (insulin growth factor-1), polypeptides and increased tau protein levels regulated by IGF receptors [65, 66]. Further studies on model organisms confirmed the impaired insulin signaling and oxidative insult of rat/mice brains leading to decreased brain size and neurodegeneration when treated with streptozotocin (STZ), a chemical which causes a diminution of insulin, and induces an insulin-resistant brain state [67, 68]. Moreover, the success of diabetic drugs to reduce AD complexities also opens new doors for researchers to understand their mechanisms in depth. In the following discussion, we review the common pathways linking AD and diabetes, and recent advancements in these fields.

Common features in Alzheimer's disease and diabetes

As described earlier, formation of Aβ and NFTs in AD, and hIAPP and insulin resistance in diabetes are characteristic hallmarks for these diseases, respectively. Both AD and diabetes are degenerative diseases involving neuronal loss and β-cells destruction, respectively [69-72]. The main systematic link between these diseases is impaired insulin signaling leading to

neurodegeneration and cognitive damages [73]. Studies have found a twofold higher risk of AD in diabetic patients [74] where diabetes was also found to accelerate the onset AD [75, 76]. Moreover, in diabetic patients with ApoE4 allele (that is genetically associated with AD); chances of AD were twofold higher than in non-diabetic patients with ApoE4 allele. ApoE4 is associated with APP trafficking, inhibition of Aβ clearance and lipid (mainly cholesterol) transportation. Insulin regulates cholesterol biosynthesis, and high-cholesterol levels were found not only to be a risk factor for diabetes but also for AD [77, 78]. Recent studies also elaborate the role of impaired insulin signaling in tau hyperphosphorylation, revealing another association between AD and diabetes [79].

AD and diabetes not only shares common pathways but also several enzymes (glutamic acid decarboxylase, dopadecarboxylase), growth factor receptors (p75 receptors, neuronal growth factor receptors and thyrotropin-releasing hormone) and second messenger abnormalities such as dysregulated protein phosphorylation and glycogen synthase kinase 3 (GSK3) over activity [73, 80-82]. Furthermore, mitochondrial dysfunctions resulting in increased oxidative stress and induced glyceraldehyde-derived advanced glycation end products (AGEs) are also associated with AD and diabetes [68, 83]. This review contains a brief outline about these common features and recent advancements in these fields.

Epidemiological/ environmental risk factors

Aging is associated with an increased risk of AD and diabetes. While aging, the decrease in mitochondrial function and oxidative capacity is observed and such is the case in AD and diabetic patients [84, 85]. In the USA, approximately one in eight persons aged 65 and older (13%), and more than 45% of people > 85 have AD. Meanwhile 42% of the population over 65 are afflicted with diabetes [86-88]. In Australia, dementia is considered as second leading cause of death. There are around half a million-population aged 65 living with dementia and expected to reach more than one million by 2056. The current care cast of dementia is around $18.7 billion that will be raised to over $36.8 billion by 2056 (https://www.fightdementia.org.au/statistics).

The aging process is frequently associated with environmental factors, especially the diet of the person. Dietary restriction or caloric restriction (CR) is linked to increased lifespan and numerous model systems showing longer life spans in CR studies. Moreover, CR has also shown to delay several age-associated diseases such as neurodegeneration, diabetes, cancer and heart disease [89-92]. Unrestricted food intake could result in obesity, which is mainly associated with excess body fat (adiposity) and excessive energy intake. Various studies found CR beneficial; recent studies on mice and Rhesus monkey found no or opposite effects of CR on lifespan. The possible

explanation for this effect was the selection of diet during lab experiments that significantly altered the genetic variations linked with life-prolonging effect in these models [89-91]. Unrestricted food intake could result in obesity, which is mostly associated with excess body fat (adiposity) and excessive energy intake. Obesity is the most highlighted risk factor linked with diabetes and AD. Obesity usually refers to higher amounts of fat in the body than regular, although the normal threshold of adiposity is not clear and is associated with body height. Whereas, increased risk of insulin resistance, diabetes, dyslipidemia, hypertension, heart diseases, cancer, and respiratory disease are associated with adiposity [93, 94]. Body mass index and waist circumference are two methods to measure adiposity. Several studies have reported higher risk of diabetes and AD with obesity and individuals with midlife body mass index > 30 have greater chances of AD [95, 96]. Increased cognitive impairment has also been reported in patients as well as in experimental models with obesity and/or dyslipidemia [97]. Furthermore, waist circumference above a cut off value (cut off value for men is 102cm and 88cm for women) is also associated with diabetes as well as AD [98].

Several epidemiological studies performed in different countries have shown the positive correlation of diabetes with AD, where diabetic patients exhibit an increased risk of AD development when compared to non-diabetic individuals. Recent population-based studies showed 2 to 5 times higher incidence of AD in patients suffering from diabetes [99-102]. Furthermore, recent studies reported AD as a form of diabetes and termed as "type 3 diabetes" due to overlapping of molecular and biochemical features that are discussed in detail later in this review. [103, 104].

Misfolding of processed amyloid peptides to form plaques

One of the characteristic features of AD is amyloidogenesis (changing of soluble protein into insoluble fibrillary protein aggregates to form Aβ plaques) in various brain regions that also pathologically represent AD [105]. Extensive study of Aβ formation and its after effects has led to the formulation of the Aβ cascade hypothesis, which places Aβ as the primary cause of AD pathogenesis [106]. This process begins with the proteolytic processing of the precursor protein into peptides predominantly of 40 or 42 amino acids ($A\beta_{1-40}$ and $A\beta_{1-42}$) [107]. These Aβ oligomers can directly inhibit the hippocampal long-term potentiating (LTP) component of memory by impairment of synaptic plasticity [108]. Ultimately, the peptides aggregate to form extracellular protein plaques (also called senile plaques) that are cytotoxic, resulting in neurological dysfunction [107]. Aβ may undergo conditional conformational changes (from native random coil structure to alpha-helical or beta-pleated strands) under slight alterations in pH, peptide concentration, metal ion concentration and environmental composition. These changes may result in diverse Aβ aggregation,

however,which confirmation is directly linked to neurotoxicity is still unknown. The neurotoxicity of Aβ is discussed later in detail with relation to other mechanisms [109, 110].

A related protein, hIAPP (human amylin), is produced in β-islet cells of the pancreas [111]. It is a 37 residues protein synergistically acting with insulin to control glycaemia [112] and regulates food intake by slowing gastric emptying, signaling the brain to decrease meal size thus reducing blood-glucose levels [113]. Additionally, it is also involved in the regulation of calcium homeostasis, vasodilation and renal filtration [81]. Damage of β-cells in diabetes resulted in deposition of misfolded hIAPP as amyloid fibrils. As hIAPP is co-secreted with insulin, insulin resistance leads to overproduction of hIAPP that accumulates as amyloid fibrils on β-cells [112, 114]. Reduced β-cells mass with the presence of β- sheet amyloid fibers of hIAPP are the characteristic of T2DM. In isolated islets, these amyloids were associated with increased apoptosis with decreased area and viability of β-cells [115].

Thus, Aβ and hIAPP both can aggregate to form extracellular plaques, but this occurs in two different organs, brain and pancreatic β-cells, respectively [111]. The distinct tissue plaque formation is associated with the unique pathology of each disease, but whether this is a causal relationship is still unknown [109]. Despite the dissimilarity in amino acid sequence, these amyloids share structural similarities more than 90%, and are believed to share common mode of toxicity [116]. A recent study by Fu et al. [117] found that Aβ and hIAPP both were involved in triggering multiple cellular pathways such as MAPK, Akt, cFos and signal transduction mediator protein kinase A by increasing cytosolic cAMP and Ca^{2+} levels in HEK293 cell line through amylin receptor-3 (AMY3). This change was blocked using AMY3 antagonist AC253. Meanwhile, in a study by Gazit group, small aromatic molecules naphthoquinones termed as NQT_{rp}^{23} and Cl-NQT_{rp}^{31} were observed as potential inhibitors of Aβ, *in vitro* as well as in fly and murine model systems of AD. Furthermore, they found that these molecules were also capable of disaggregate amyloid formed by hIAPP with unknown mechanisms [118]. These results not only strengthen the AD-diabetes association but also point out new therapeutic target for AD and diabetes. Moreover, human Ca^{2+} binding protein nucleobinding 1 (NUCB1) was found to be useful in inhibiting hIAPP fibril formation as well as disaggregation of pre-existing hIAPP fibrils through unknown mechanism [119]. We propose that NUCB1 should be checked as a potential inhibitor of Aβ plaque formation in AD patients.

Impaired insulin and glucose metabolism in AD and diabetes

Insulin is a hormone that has an important role in glucose homeostasis in a dynamic relationship with both feeding and fasting as well as in growth and development of body tissues [120, 121].

Insulin is mainly synthesized by pancreatic β-cells and secreted to the peripheral circulatory system. Although insulin is transported into the brain through the blood-brain barrier (BBB), evidence indicates that the brain can synthesize insulin locally [122]. In the central nervous system, insulin also normalizes and maintains cognitive functions by regulating key processes such as neuronal survival and longevity, learning, and memory. Defective insulin signaling leads to energy deficient neurons resulting in various metabolic insults and impaired synaptic plasticity, which are discussed later [122, 123].

Insulin resistance in peripheral system and central nervous system

Insulin resistance may be defined as a diminished ability of cells or tissues to respond to physiological levels of insulin. Insulin resistance can be associated with defects in insulin receptor function, insulin signal transduction, glucose metabolism, transport, glycogen synthesis, hyperinsulinemia, hyperglycemia, inflammation and lipid's metabolism [124]. Increased lipid content induces insulin secretion referred to as hyperinsulinemia for maintaining normoglycemia. Any defects in insulin secretion (low insulin levels, type 1 diabetes) and signaling (insulin resistance at cellular levels, type 2 diabetes) may cause hyperglycemia or high blood sugar levels. In this situation diabetes, is a disease originating from defects in the body's ability to control insulin and glucose homeostasis due to hyperglycemia, insulin resistance and β-cell failure [125, 126]. Insulin resistance also has a pivotal role in the progression of neurodegenerative diseases, especially in AD. Many studies have found neuro-protective effects of insulin [67, 127, 128]. Insulin deficiency, resistance and hyperinsulinemia are involved in cognitive impairments observed in patients with diabetes [129] . AD is associated with reduced insulin and insulin mRNA as well as decrease level of insulin receptors [66]. *In vitro,* studies have shown the impaired insulin signalling, hyperphosphorylated tau proteins, and neural loss in T2DM affected AD animal models [129-132].

Increased cerebrospinal fluid (CSF) insulin levels are correlated with high peripheral insulin in AD patients [71]. Insulin also has effects on Aβ metabolism. Hyperinsulinemia can increase extracellular Aβ concentration by stimulating its trafficking from Aβ generating sites (trans-Golgi network and endoplasmic reticulum) that results in reduced intracellular concentrations of Aβ. Insulin degrading enzyme (IDE) plays an important role in Aβ degradation. Insulin can competitively inhibit IDE that results in reduced Aβ degradation [133, 134]. Induced activity of IDE in transgenic mice brain results in decreased levels of Aβ thereby preventing cognitive decline [135]. Furthermore, persistent peripheral hyperinsulinemia leads to decreased transportation of insulin to the brain and causes an insulin-resistant brain state resulting in reduced insulin levels in CSF [65]. Brain insulin resistance consequently decreases the IDE levels in AD patients resulting in increased Aβ neurotoxicity [136]. Interestingly, Aβ also can reduce insulin signaling and receptor

auto-phosphorylation suggesting that Aβ competitively inhibits insulin binding. Moreover, Aβ is also reported to reduce insulin receptors substrates signaling in cultured cells [133, 137]. These findings suggest an important role of insulin dysfunction in neurodegenerative diseases.

Insulin signaling mechanism

Insulin implements its actions through insulin signaling pathway by interacting with insulin receptors. A brief outline is presented here. Preproinsulin, an inactive single chain precursor with a signal sequence, is the primary form of insulin secreted by pancreatic β-cells.

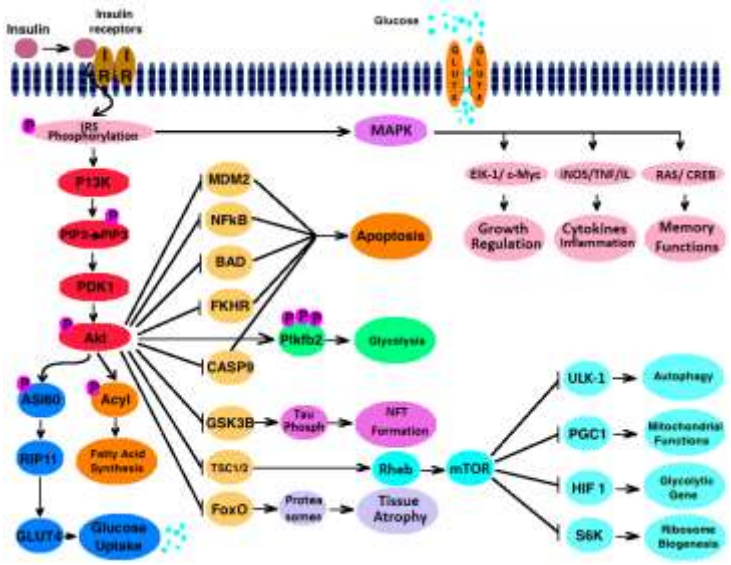

Figure 2.1: Linking insulin-signaling mechanism with diabetes and AD. Impaired insulin signaling due to insulin resistance or defects in insulin receptors and Akt phosphorylation lead to activation of all pathways that remain blocked during normal signaling. Deactivation of Akt triggers several transcription factors that resulted in abnormal cell functions, apoptosis, neuronal cell death via NFT production, tissue atrophy and inflammation. These are major hallmarks in patients with AD and diabetes.

After removal of the signal sequence by proteolysis, preproinsulin changed to proinsulin that is further converted to insulin by special proteases when blood glucose or amino acid concentration increases. Active insulin consists of two chains, bound together with disulfide bonds [138]. Insulin performs its duty by binding to its specific receptors known as insulin receptors (IR). Insulin binds to the α-subunit of the receptor and activates the tyrosine phosphorylation of the β-subunit of the receptor [139, 140]. This process activates two important signaling pathways: Akt (also known as protein kinase B: PKB) and MAPK (mitogen activated protein kinase) [141, 142]. Akt signaling regulates cell growth, proliferation, survival, and protein synthesis, while MAPK signaling activates cell differentiation, proliferation and death (Figure 2.1).

Insulin signaling in diabetes and AD

Activation of Akt results in phosphorylation of the 3'-OH group of inositol thus generating PIP2 and PIP3 (phophoinositide phosphates) that further activates serine/threonine kinases, 3'-

phosphoinositide-dependent-kinase-1 (PDK-1), PKB/Akt, and MAPK regulatory pathways [143, 144]. As Akt and MAPK are major pathways involved during insulin signaling through tyrosine phosphorylation on insulin receptors, defects in insulin receptors, insulin receptor substrates (IRS) and these pathways lead to complications that are hall mark of diabetes, AD and their related abnormalities [145, 146].

Defects in insulin receptors

A decrease in tyrosine kinase activity of insulin receptors was observed in T2DM suggesting a post-receptor defect with reduced association of the insulin receptor with p85 subunit of Akt leading to impairment of insulin stimulating glucose disposal and glycogen synthesis [147]. This also leads to defects in glucose transportation and lower level of GLUT4 mRNA in muscles. Glucose may undergo glycolytic pathway or glycogen synthesis after phosphorylation through hexokinases (HK-I to IV) [126, 148, 149]. Increased insulin induces glycogen synthesis, while decreased glycogen synthesis in muscle cells due to insulin resistance is a hallmark in T2DM, especially in skeletal muscles [126, 150]. Rare mutations on IRS, specifically IRS-2 may lead to insulin resistance in T2DM patients as chances of diabetes were more in IRS-2 mouse mutants compared to IRS-1 [151]. Insulin receptors found in the brain's hippocampus, and cerebral cortex play an important role in memory and learning [66, 152]. APP mutated mice lacking IRS-2 show induced premature mortality and Aβ deposition lead to the IR role in AD pathology [153, 154]. A reduced insulin signaling was observed in brains of AD patients. Aβ also has the ability to induce insulin resistance by down regulating the IRS expression [133].

Akt signaling

The binding of Akt with insulin receptors is affected by serine phosphorylation of IRS that decreases the tyrosine phosphorylation of IRS due to several mechanisms such as hyperglycemia, inflammation, hyperinsulinemia, hyperlipidemia and mitochondrial dysfunction. It may lead to disassociation of Akt from IRS or IRS from insulin receptors [155, 156]. Circulating free fatty acids (FFA) and tumor necrosis factor (TNF) may induce serine phosphorylation in muscle cells leading to IRS dysfunction [157, 158]. Although the mechanism of insulin signal impairment through FFA is still unclear, the intracellular lipid metabolites such as diacylglycerol (DAG) and acetyl CoA lead to insulin resistance through IRS serine/ threonine phosphorylation via protein kinase C (PKC) and may result in endothelial dysfunction in T2DM [159, 160]. Diacylglycerol that induced the PKC is also observed in muscles during lipid infusions and fat feeding. FFA also induces the PKC phosphorylation independent of PDk1 and translocates PKC to the nucleus and inhibits IR gene transcription [161, 162]. A recent study by Liu et al. has observed the decreased levels of insulin signaling components, increased hyperphosphorylation of tau and reduced O-GlcNAcylation (O-

GlcNAc) levels in the brain of patient suffering from T2DM. Decreased brain glucose levels are resulted in reduced O-Glena levels that regulate phosphorylation of tau inversely. Interestingly, O-Glena modification also regulates the AKT phosphorylation [163, 164]. It is interesting to know that at first insulin induced IDE expression in hippocampal neurons, while IDE decrease is associated with reduction of Akt p85 subunit. This observation lead to the hypothesis that IDE reduction by insufficient insulin might increase the chances of AD, as brain IDE activity reduced in hyperinsulinemia conditions [165, 166].

Akt signaling may be blocked by PTEN (phosphate and tensin homologue deleted on chromosome ten) and tumor suppressor protein phosphatase resulting in dephosphorylation of PIP2 and PIP3 [167, 168]. Imbalances among the Akt subunits p85 and p110, and their heterodimer p85-p110 are also responsible for insulin resistance. Akt p85 unit competes with p85-p110 (as this heterodimer unit is responsible for Akt activity), and the p85 induction is caused by human placental growth hormone. This type of resistance is observed in pregnant women with T2DM [169-171]. Akt triggers downstream effects leading to glycogen synthesis, hepatic glucose production and on/off switch of many transcriptional factors. Studies in mice have shown that deletion of Akt leads to insulin resistance. Upon activation, Akt blocks apoptosis-related genes such as BAD, caspase 9, GSK3, FoxO family transcription factors, CREB and NF-kB. It also inhibit the MDM2 activity by blocking p53 [166, 172]. Furthermore, Akt phosphorylation allows the translocation of GLUT4 (glucose transporter) to the plasma membrane by AS160 phosphorylation [173]. The mutated Akt was unable to phosphorylate downstream targets and inhibition of gluconeogenic through phosphoenolpyruvate carboxykinase (PEPCK) [174, 175]. Another factor regulating gluconeogenesis and its related genes PEPCK and G-6-pase are FoxO protein 1 and 3 in patients with hyperglycemia. A reduction in Akt activity leads to decreased phosphorylation on FoxO allowing its entrance to the nucleus and activation of its transcription factors in T2DM. Phosphorylation of FoxO regulates gene transcription and inhibits apoptosis to promote neural growth and survival through phosphorylation of BAD (Bcl-2 associated death promoter protein) [176-178].

Akt also initiates protein synthesis by p70s6k phosphorylation that may shutdown IRS activity by direct phosphorylation of IRS through mTOR (overactive mammalian target of rapamycin) signaling triggering by Rheb (GTPase RAS homolog enriched in the brain).). Akt also regulates the binding of tuberous sclerosis protein 1 and 2 (TSC1 and TSC2) (). Upon phosphorylation, TSC2 is released and activates Rheb and mTOR pathway [179, 180]. mTOR further activates initiation factor 4E-BPs (4E-binding proteins) phosphorylation and inhibits its

binding with elF (eukaryotic initiation factor) and promotes elF4E cap-dependent translation that controls cell growth and survival. This event also activates adipocyte differentiation transcription factors, PPARγ, CCAAT enhancer binding protein α (E/EBP) etc. that leads to adipogenesis, obesity and insulin resistance if not properly controlled [181-184]. Activation of p70S6 protein regulates ribosomal function by phosphorylating ribosomal proteins and initiates amino acid addition to newly synthesized peptides. In T2DM, effected kidney tissues, induced mTOR levels were observed with matrix expansion and renal hypertrophy [185, 186].

Under normal conditions, Akt signaling phosphorylates the glycogen synthase kinase 3B (GSK3B) and inactivates glycogen synthase. Insulin resistance leads to dephosphorylation and activation of GSK3B. Increased expression of GSK3 was observed in patients with neurodegeneration and was associated with Tau hyperphosphorylation [187, 188]. A study by Schubert reported high tau phosphorylation with significantly reduced phosphorylation of Akt and GSK3 in NIRKO mouse brains [189, 190]. Hyper tau phosphorylation via GSK3 impairment is associated with NFT formation within the brain, which is an important hallmark of AD [191].

In brief, defects in Akt signaling not only lead to defects in glycogen synthesis, gluconeogenesis, glucogenolysis, and amino acid synthesis but also promote AD through hyperphosphorylation of tau and reduction of O-GlcNAcylation.

MAPK signaling in diabetes and AD

MAPK signaling activates intracellular enzymes that can respond to stimuli from extracellular environment. MAPK pathway runs parallel to Akt and stimulates extracellular signal-regulated kinases 1/ 2 (ERK1/2) that induce activation of several transcription factors such as ElK-1 and c-Myc that are important in cell growth regulation [192, 193]. The Ras-ERK/MAPK activated cascade is thought to be involved in memory formation by synaptic plasticity [194]. MAPK signaling also activates MK2 (MAPK-activated protein kinase 2) that is further involved in mediating the inflammatory response to cellular stress. It is shown by cell culture studies that MAPK activation resulted in expression of inflammation-associated iNOS, TNFα and IL-1β genes [195]. MAPK activation also up-regulates cytokine production by direct phosphorylation of transcription factors such as CREB (cAMP response element binding). CREB has a direct effect on the structural changes associated with memory formation [196] .

MAPK pathway is involved in excessive tau phosphorylation, neuro-inflammation and synaptic plasticity in AD patients. Induced MAPK expression is reported in hippocampal and cortical regions within the brain in AD patients when compared with aged-matched healthy individuals. Furthermore, greater immune-reactivity of MAPK is observed in AD post-mortem

brains [197, 198]. Moreover, in transgenic mice, tau hyperphosphorylation was found to be directly associated with phosphorylated MAPK. In addition, Aβ fibrils in microglia also able to activate MAPK signaling that resulted in inflammatory gene expression and elevated pro-inflammatory cytokines [199]. Briefly, impaired MAPK signaling is not only associated with neuro-inflammation but also leads to the promotion of Aβ and NFTs generation.

Abnormal glucose metabolism

Abnormal glucose metabolism is a characteristic of T2DM and is mechanistically linked with AD [111]. Metabolic abnormalities due to impaired glucose consumption and energy metabolism in AD almost resemble with T2DM. In addition, infirm glucose metabolism and insulin resistance also lead to memory and synaptic dysfunction in patients suffering from diabetes (Figure 2.2) [200, 201].

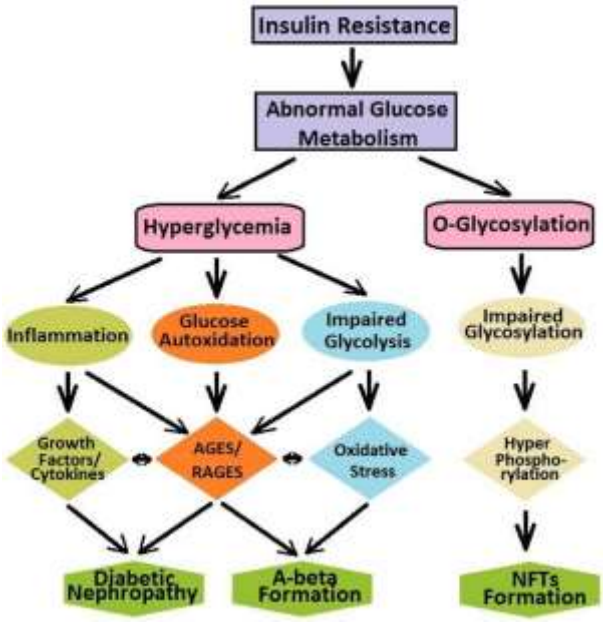

Figure 2.2: Hyperglycemia-prompted activation of molecular pathways associated with AD, and diabetes. Defects in insulin signaling pathway and/ or in production of insulin lead to hyperglycemia. Hyperglycemia promotes production of AGES, oxidative stress, and induces levels of various growth factors and cytokines that accumulatively endorse the AD and diabetic complications.

Furthermore, a decrease in cerebrospinal fluid (CSF) insulin was reported in AD patients [71]. These findings lead to the conclusion that normal glucose metabolism is associated with standard cognitive response and any defects in the glucose metabolism lead to perceptive system decline. Brain neurons are unable to produce and store glucose, and require continuous glucose transportation through the blood-brain barrier (BBB) by glucose transporters (GLUTS). Glucose transporters, isoforms 1, 3, 4 and 8 are abundant in the brain. GLUT 1 is expressed on BBB endothelial cells and cortical membranes, GLUT 3 is expressed on neurons while GLUT 4 and

GLUT 8 are expressed in intracellular compartments of neurons [68, 202, 203]. Reduced levels of GLUT1 observed in AD patients as well as in transgenic mice lead to reduced supply of glucose from the peripheral transport system [8]. Reduced GLUT 1 and 3 levels and decreased glucose utilization are likely to be associated with tau hyperphosphorylation, increased density of NFTs, and reduction in O-GlcNAcylation [204-206].

Functional neuro-imaging technique positron emission tomography (PET) has been found best to monitor neuronal activity and specific biological processes at tissue levels *in vivo*. PET with 2-[^{18}F] fluro-2-deoxy-D-glucose (FDG) is used to estimate qualitative and quantitative changes in cerebral metabolic rate of glucose (CMRglc) [207]. Several studies have reported the cerebral metabolism decline before the attenuation of cognitive functions. In AD, reduced, CMRglc has been reported in hippocampus, parietal-temporal and prefrontal regions within the brain when compared with age-matched health individuals [208, 209]. Decreased CMRglc metabolism may lead to mitochondrial dysfunction, hyperglycemia and inflammation within the brain. A 55-65% decrease in cerebral glucose utilization was observed not only in AD patients and in transgenic models but also in individuals carrying the apolipoprotein 4 alleles. ApoE4 allele can lead to reduced glucose metabolism and energy production within the brain [80, 210, 211]. Reduced activity of mitochondrial proteins mainly associated with TCA cycle such as α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase were observed in AD patient's brain tissues. This reduced activity was resulted in the release of cytochrome c from mitochondria, and caspase-3 activation leading to neuronal cell death. Moreover, these changes were positively associated to the degree of clinical disability in AD [212, 213]. Furthermore, 50% decrease in ATP production from glucose was also reported in early AD. All these findings suggest that ATP-dependent energy generation is vital for normal cell performance [214].

Although glucose is the least reactive reducing sugar, it may lead to non-enzymatic Amadori products through Schiff base formation by reacting with free amino groups of proteins, lipids and nucleic acids. These Amadori products accumulate on proteins and initiate the process of advanced glycation [215, 216]. Hyperglycemia resulting from insulin resistance promotes the production of foremost glycation end products (AGEs) that are involved in AD pathogenesis. Although this process occurs during normal aging, it becomes highly accelerated in diabetes. AGEs are accepted as active contributors in AD progression and induce Aβ and tau glycation that lead to Aβ aggregation and NFTs formation in the brain [217, 218].

AGEs implement their functions and induce several biological processes through binding with RAGEs (receptors for advanced glycation end products). RAGEs not only interact with AGEs

but also bind and interact with Aβ. Activation of RAGEs through binding with these ligands may initiate ROS production and inflammation [219, 220]. Levels of RAGEs were increased in RAGE-bearing cells from AD patients when incubated with Aβ that resulted in increased oxidative stress through NADPH oxidase-like mechanism, and activated NF-kB pathway [221]. Furthermore, Aβ transportation across the BBB was associated with RAGE that was blocked by using anti RAGE IgG or sRAGE (soluble RAGE). Moreover, RAGE associated transport of Aβ and induction of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and heme oxygenase type 1 was also observed in mice neuronal cells. Mice administrated with sRAGE showed decreased Aβ production and accumulation in brain parenchymal regions [222].

It is suggested that vascular remodeling in diabetes and AD linked cerebrovascular amyloid angiopathy (CAA) are both associated with RAGE expression [223]. Aβ-RAGE interaction also has effects on cerebral blood flow in murine models. Infusion of Aβ into peripheral circulation resulted in reduced cerebral blood flow that was prevented by sRAGE, RAGE IgG or RAGE specific inhibitor (FPS-ZM_1) administration [222, 224]. Furthermore, induced levels of vasoconstrictor mediator endothelin-1 were induced to block Aβ-associated suppressed cerebral blood flow. A recent study by Xiong F et al. reported that RAGE expression in the human umbilical vein endothelial cell line (ECV-304) had prevented the endothelial cell membrane repair by activating β-catenin levels that resulted in reduced F-actin stress fibers and attenuated plasma membrane resealing [225].

Impaired glucose metabolism also plays a very important role in NFTs formation by promoting hyperphosphorylation of tau protein. Tau protein undergoes many posttranslational modifications (PTMs) particularly phosphorylation and glycosylation. Tau phosphorylation has been thought to inhibit the binding of tau protein from microtubules, while hyperphosphorylation leads to self-aggregation of tau filaments and NFTs formation. *O*-glycosylation (addition of one molecule of N-acetylglucosamine to serine or threonine residue) is thought to regulate tau phosphorylation reciprocally by competing phosphate groups on single or proximal Ser/ Thr residues [226, 227]. Although the exact mechanism by which *O*-glycosylation regulates tau phosphorylation is unknown, *O*-glycosylation is thought to be associated with glucose metabolism. STZ-induced diabetic rat showed reduced *O*-glycosylation levels that were restored after insulin injection [228]. Decreased GLUTs levels and *O*-glycosylation had also been reported within the AD brain when compared to normal [79, 229]. A recent study by Yuzwa SA et al. has observed that *O*-glycosylation at tau protein not only delays neurodegeneration but also hinders tau oligomerization [230]. These observations suggest *O*-glycosylation as a potential therapeutic target to slow down

AD progression and this mechanism also provides evidence of shared pathology between diabetes and AD.

Inflammation

Insulin resistance in diabetes and AD also leads to inflammation. Vascular inflammation mediated by RAGE has been proposed as a possible mechanism for vascular dysfunctions in AD and diabetes. Furthermore, the levels of inflammatory mediator's IL-6 and c-reactive proteins were raised in diabetic, and AD patients [231]. It is reported that diabetes accelerates memory dysfunction via promoting Aβ aggregation and cerebrovascular inflammation by up regulating RAGE in diabetic mice AD models [60]. Srikanath et al. and Ramasamy et al. extensively reviewed the role of AGEs and RAGE in development of AD. Briefly, RAGE can be activated by many ligands as described earlier. AGEs and Aβ synergistically induce the expression of the pro-inflammatory cytokines IL-6, TNF-α and M-CSF (macrophage colony-stimulating factor). Aβ mediated persistent activation of microglial cells due to pro-inflammatory state is resulted in neuronal cell death and disease progression in AD. Interestingly, inflammation induced levels of both Aβ and AGEs. Stimulation of TNF-α accelerates BACE1 expression and resulting in enhanced amyloidic processing of APP in astrocytes and formation of Aβ in diabetic models of AD [232, 233].

Summary of the section

Impaired insulin signaling and glucose metabolism are not only risk factors for diabetes but also for AD. Peripheral insulin dysfunctions induce impairments to insulin functions in brain that result in Aβ aggregation and NFTs formation. Furthermore, abnormal glucose metabolism not only promote oxidative stress, Aβ aggregation and NFTs formation but is also responsible for vascular angiopathy and inflammation in AD as well as in diabetes. Additionally, AGEs and RAGE both promote AD and diabetic complications and provide a common link between these two diseases. In the wording of Kroner et al. “co-existence of brain insulin deficiency and resistance suggests that AD might be form of diabetes i.e. type 3 diabetes” [234].

Linking lipids with AD and diabetes

Both AD and diabetes are not only characterized by insulin metabolism impairments but also by dyslipidemia. Lipid-mediated signaling regulates many physiological processes like trafficking and proteolytic activities of membrane-bound protein [235, 236]. Fatty acids (FAs) are mostly synthesized in all body cells, stored and circulated as triglycerides. FAs can be degraded by lipases such as phospholipase (PL) and lipoxygenase (LOX), and re-synthesized in the form of phospholipids [237]. Increased levels of FFAs, triglycerides, cholesterol and low-density

lipoproteins (LDL), and reduced levels of high-density lipoproteins (HDL) are well described risk factors not only for diabetes but also for neurodegenerative diseases like AD [238-241]. Induced FFAs levels in brain were also linked with mitochondrial dysfunction, and IDE inhibition that resulted in increased Aβ production [242, 243].

Phospholipase isoform A2 (PLA2) hydrolyses the phospholipids and converts them to arachidonic acid (AA) which further can be converted to various signaling molecules required for cellular metabolism [237]. Several studies have found that induced levels of LOX and PLA2 are associated with higher intensity of Aβ, and cognitive decline in transgenic mice models [244, 245]. Furthermore, AA also activates NADPH, a major source of ROS production in neurons that consequently activates cytoplasmic PLA2 (cPLA2) [246, 247]. cPLA2 regulates AA release at synapses. In neurons, AA is involved in synaptic functions, and it's over production can trigger depolarization of neuronal cells via calcium dependent apoptosis [248]. FAs metabolism through LOX also produces ROS and is very sensitive to glutathione (GSH) a major antioxidant in mitochondria. GSH can be depleted under high concentrations of LOX [249]. A reduction in Aβ production has been reported in LOX deleted transgenic mice [244]. Moreover, LOX metabolites such as 5-hydroperoxy eicosatettraenoic acid and leukotriene have been reported to positively induce Aβ production in cultured cell lines [250]. AA can also be converted to inflammation mediators, eicosanoids, and prostaglandins by the action of cyclooxygenase (COX) [251]. Elevated levels of PL2, LOX and COX are reported in patients with AD and diabetes [252]. These data suggest that diabetes can mediate AD through lipid metabolism as increased FFAs levels are reported in diabetes and derivatives of AA may be responsible for Aβ production in AD [191].

Hypercholesterolemia also contributes to the pathology of AD and diabetes. Hypercholesterolemia is associated with increased risk of type 2 diabetes and more than 70% patients diagnosed with type 2 diabetes had hypercholesterolemia. High levels of cholesterol and other fatty acids lead to arthrosclerosis and vascular lesions [253, 254]. In the brain, unesterified cholesterol is present in cellular membranes and myelin sheets of astrocytes and neurons. As the majority of lipoproteins cannot cross BBB, cholesterol in the brain is derived from de novo synthesis [255]. In the brain, excess cholesterol is converted to cholesteryl esters by enzyme acyl CoA: cholesterol acyltransferase 1 (ACAT1) or oxysterol 24S-hydroxycholesterol (24OHC). 24OHC can be easily transported to the peripheral circulation through the BBB. Moreover, 27S-hydroxycholesterol (27OHC) which is produced outside the brain, can cross the BBB and is reported to increase in AD patients [256-258]. Brain lipoproteins that transport lipids resemble HDL particles present in cerebrospinal fluid and apolipoprotein E is the most studied lipid carrier in AD

pathology discussed later [259]. Induced levels of ACAT1 associated with increased Aβ generation and vice versa in mouse models suggests direct association of cholesterol esters with Aβ production and AD promotion [260, 261]. Furthermore, cholesterol also modulates the BACE1 and secretase activities. Reduction in cholesterol levels lead to decreased activities of BACE1 and secretase that resulted in reduced Aβ production. Moreover, association of BACE1 with lipid rafts is found to be positively linked with amyloidic processing of APP; and production of Aβ is decreased after cholesterol depletion [262, 263].

ApoE is a well-established risk for the late-onset AD. ApoE that is significant for lipid transport also plays an important role in Aβ clearance as well as stimulation of IDE which is also involved in Aβ degradation [264]. The ApoE3 allele is most common with 77% occurrence followed by an ApoE4 (15%). The ApoE4 allele is significantly higher in AD patients, and common individuals carrying the ApoE4 allele have increased risk of developing AD when compared to non-carriers [253]. Furthermore, diabetic patients who are ApoE4 allele carrier have two-fold more chances to develop AD than usual diabetic individuals [265]. Aβ clearance in the brain is ApoE isoform specific (ApoE4< ApoE3<ApoE2). High levels of ApoE4 were associated with induced Aβ generation [266]. Moreover, transgenic over expression of ApoE4 in neurons increases the tau phosphorylation suggesting that ApoE4 is also involved in development of NFTs by unknown mechanism [267]. Diabetes and ApoE4 synergistically increase the risk for AD. Various studies linked type 2 diabetes to brain pathology, particularly in individuals with ApoE4 allele (reviewed by Luchsinger et al.) [268]. Low lipid clearance characteristic of ApoE4 may also make it an independent risk factor for type 2 diabetes. A current study by Chaudhary et al. has found that ApoE4 allele has influence on lipid plasma levels and is associated with diabetes with or without coronary artery disease [269].

Intracellular cholesterol trafficking is mainly thought to be modulate by ATP-binding cassette (ABC) transporter family proteins such as ABCA [270]. ABCA1 primary function is the transportation of cholesterol efflux onto lipid-poor ApoE. Mutations in human ABCA1 allele result in Tangier disease (absence of HDL in plasma with increased cardiovascular risk) and cause significantly low levels of ABCA1 and ApoE in the brain [271]. ABCA1 deficiency in mice led to 80% decrease in the brain ApoE levels.

Thus the poor lipidation of ApoE due to decreased ABCA1 levels is resulted in increased Aβ production and vice versa [268]. A recent study by Wang et al. has reported high-cholesterol levels in the STZ-induced rat brain with decreased expression of ApoE mRNA. However, they have observed decreased cholesterol levels and high ABCA1 expressions in peripheral tissues [269].

These results suggest that in case of diabetes, the body tends to decrease cholesterol levels by expressing ABAC1. Moreover, diabetes also results in high-cholesterol levels within the brain. poor lipidation of ApoE due to decreased ABCA1 levels is resulted in increased Aβ production and vice versa [272]. A recent study by Wang et al. has reported high-cholesterol levels in the STZ-induced rat brain with decreased expression of ApoE mRNA. However, they have observed decreased cholesterol levels and high ABCA1 expressions in peripheral tissues [273]. These results suggest that in case of diabetes, the body tends to decrease cholesterol levels by expressing ABAC1. Moreover, diabetes also results in high-cholesterol levels in the brain.

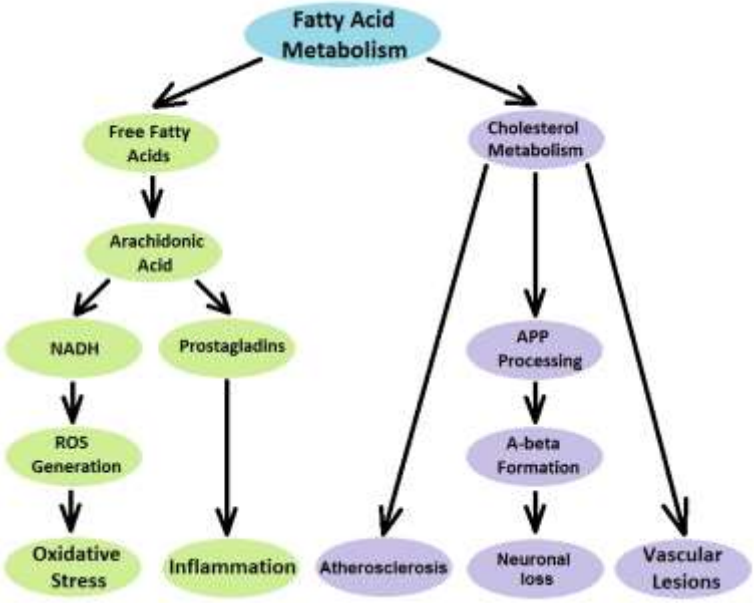

Figure 2.3: Lipid metabolism in AD and diabetes. Production/ accumulation of excessive free fatty acid and cholesterol lead towards several complications like oxidative stress, inflammation, arthrosclerosis, vascular lesions, and neuronal loss that are major hallmarks of these diseases.

Previous studies suggested that impaired lipid metabolism is as important as insulin dysfunctions in AD and diabetes. Although much attention is given to ApoE, lipid metabolism association between AD and diabetes has not been fully addressed to date. More research is required to explore the role of fatty acids in the formation of Aβ plaques and NFTs in AD, as this mechanism is still unclear. Figure 2.3 represents a way how impaired fatty acid metabolism may be responsible for diabetes and AD promotion. Secondly, very limited studies are available on any correlation between human amylin and ApoE in diabetes as well as in AD. Although one study found that amylin fibrillation could be prevented by lowering the ApoE4 levels in T2DM [274], no further evidence came from this side.

Mitochondrial dysfunctions

Aerobic organisms mostly produce energy into the form of ATP utilizing oxygen and nutrients. Mitochondria produce over 90% of cellular ATP through oxidative phosphorylation, which is also the major source of ROS production. Furthermore, mitochondria also regulate other physiological processes and cellular functions like cell survival and death, intracellular calcium homeostasis, cell cycle regulation and synaptic plasticity [275, 276]. Both AD and diabetes are associated with mitochondrial dysfunctions. Especially as neurons require high energy to perform their functions, limited glycolytic activity of neuronal cells makes them highly dependent on mitochondrial energy production, and any changes associated with mitochondrial impairment led to the neuronal dysfunction and neurodegeneration [277, 278]. Mitochondrial abnormalities like oxidative stress and impaired calcium homeostasis are reported in AD and diabetes.

Oxidative stress

According to Perez-Matute "living with the risk of oxidative stress is a price that aerobic organisms must pay for more efficient bioenergetics" [278]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important oxidants in living organisms. These ROS produced can be converted to many forms and can permeate through inner and outer mitochondrial membranes to enter the cytoplasm. However, most ROS produced in mitochondria can be converted into water or oxygen in the presence of metal-dismutases either in mitochondria or in the cytoplasm as described later [279, 280]. The oxygen-derived pro-oxidants (ROS) can be classified under two groups; radicals and non-radicals. The radicals are superoxide ($O^{\cdot}{}_2^-$), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HO^{\cdot}{}_2$) and nitric oxide ($NO^{\cdot}$). The reactive, oxygen-containing non-radicals include hydrogen peroxide (H_2O_2), organic peroxides (ROOH), aldehydes (HCOR), hydrochlorous acid (HOCL), and peroxynitrite (ONOOH/ $ONOO^-$) [281-284].

Several hypotheses have been proposed to explain the role of oxidative stress in AD and diabetes pathophysiology, while the combination of high-energy demand, relatively low antioxidant levels and a high concentration of iron makes the brain, particularly vulnerable to oxidative stress [281, 285]. In AD pathology decline in synaptic activities and defects in low energy metabolism with comparatively increased amount of ROS are observed [286, 287]. Moreover, reduced levels of antioxidants enzymes like Cu/Zn SOD, glutathione (GSH) and catalase are found in the frontal and temporal cortex. Furthermore presence of Aβ and NFTs lead to mitochondrial dysfunctions, generation of oxidative stress and neuronal cell death [137, 288-290]. Meanwhile, diabetic patients exhibit a number of oxidative stress markers, including F_2-isoprostane and nitrotyrosine in urine, plasma and tissue, leading to the hypothesis that oxidative stress is also a hallmark of diabetes [291,

292]. Although mitochondria are capable of generating ROS/ RNS by themselves however, in AD and diabetes, other sources/ mechanisms like misfolded proteins (Aβ and hIAPP), accumulated NFTs (tau plaques), hyperglycemia and AGEs also promote ROS generation [115, 293, 294]. We will briefly discuss the generation of ROS by these sources.

Generation of oxidative stress by the mitochondrion itself

Many authors have reviewed the production of ROS and RNS in mitochondria in detail [295-299]. Here we briefly describe the ROS/ RNS generation in mitochondria. In total, nine different types of enzymes in mitochondria have the capacity to produce ROS by converting molecular oxygen to either superoxide anion or hydrogen peroxide. Cytochrome b5 reductase and monoamine oxidases (MAO) are located on outer mitochondrial membrane (OMM) whereas dihydroorotate dehydrogenase (DHOH), α-glycerophosphate dehydrogenase (α-GDH), succinate dehydrogenase (SDH), aconitase, α-ketoglutarate dehydrogenase complex (KGDHC), complex-I and complex-III are present on inner mitochondrial membrane (IMM). MAO, DHOH, α-GDH and KGDHC produce H_2O_2 via direct or indirect biochemical reactions, while Cytochrome b5, complex-I and complex-III produce super oxides. Aconitase localized in the mitochondrial matrix generates hydroxyl radical upon oxidation of its iron-sulphur cluster by superoxide. PDHC and KGDHC have also been reported to generate superoxide and hydrogen peroxide when isolated and purified from the bovine heart, and mouse brain mitochondria.

Generation of oxidative stress by Aβ, tau and hIAPP

Aβ insertion in mitochondria has been reported to disrupt the electron transport chain, reducing energy production and increased ROS production [300]. Reduced activity of complex-IV in AD patients is well documented and Aβ is believed to inhibit the functions of complex-IV. Aβ has been shown to produce hydrogen peroxide (H_2O_2) and release thiobarbituric acid reactive substances (TBARS) mainly associated with hydroxyl radicals (OH) via metal ion reduction [301, 302]. Furthermore, Aβ can also act directly on mitochondria. Aβ plaques interact with Aβ binding alcohol dehydrogenase (ABAD) resulting in increased mitochondrial membrane permeability and reduced activities of respiratory proteins [303-305]. The oxidation of proteins at lysine, arginine, proline and histidine residues via peroxynitrite generates protein carbonyls and nitrile, both of which cause an increase in the NFTs, non-tangle bearing neurons and glia of AD patients [303].

Even though, several studies report the association of Aβ and oxidative stress, few studies have looked at the role of hIAPP. However, two studies have reported the elevation of oxidative stress markers using immortalized beta cell lines when treated with hIAPP exogenously [306, 307]. Furthermore, β-cells of patients with diabetes have shown higher oxidative stress with increased

levels of hIAPP. A study by Zarika et al. reported that amyloid deposition on mice islet is associated with increased ROS levels and beta cell apoptosis [115]. Although treatment with antioxidants prevents ROS generation, it does not reduce amyloid formation. In contrast, amyloid inhibition reduces ROS generation as well as beta cell apoptosis. This information leads to the conclusion that hIAPP also induce oxidative stress and apoptosis, although the exact mechanism is still unknown.

Hyperglycemia induced ROS generation

Hyperglycemia due to insulin impairment in diabetic patients is associated with many biochemical pathways, including glucose mediated ROS production, protein kinase activation, formation of AGEs and cytokine secretion [293]. In glucose autoxidation, glucose forms enediol radicals and is converted to reactive ketoaldehydes and superoxide; consequently, hydroxyl radicals are produced in the presence of transition metals via H_2O_2 if not degraded by catalase or glutathione peroxidase [308, 309]. Hyperglycemia drives the inner mitochondrial membrane potential upward through generation of excessive electron donors in the Kreb's cycle [310]. Hyperpolarization of the mitochondrial internal membrane leads to an increased ATP/ADP ratio, which inhibits electron transport to complex-III leaving coenzyme Q in a decreased state. Coenzyme Q can pass electrons to molecular oxygen to generate the partially reduced derivative, superoxide [215, 311]. This superoxide overproduction causes a 66% decrease in GAPDH (glyceraldehyde-3-phosphate dehydrogenase) activity, PARP (poly ADP ribose polymerase) activation, and NAD^+ depletion [312]. Overproduction of superoxidase radicals is countered by superoxide dismutases (SODs) and by uncoupling proteins (UCPs). In hyperglycemia, over expression of uncoupling proteins (UCPs) blocks glucose induced cell death by preventing mitochondrial hyperpolarization and ROS formation [313, 314]. Hyperglycemia was found to be responsible for the cognitive decline in diabetic patients. Impaired antioxidant system with increased oxidative/ nitrosive stress in hyperglycemic conditions has been observed throughout the brain of STZ diabetic rats. Authors have also reported about reduced activity of ETC complexes III, IV and V, and ATP synthesis [315]. Moreover, in another study, 12 weeks older STZ-induced diabetic rat also showed lower ATP contents and calcium accumulation ability. A recent study by Cardoso et al. and Raza et al. also has reported increased ROS/ RNS levels, reduced antioxidant activity and decreased activities of mitochondrial enzymes such as complex III, complex IV in cortical and hippocampal mitochondria as well as the pancreas of STZ induced diabetic rats [316, 317].

AGEs induced ROS generation

AGEs that are found in senile plaques also produce free radicals by chemical oxidation and degradation, by binding with their receptors (RAGE) or interacting with microglia that surrounds the senile plaques and results in respiratory blast and production of ROS and RNS. AGEs particularly produce super oxides and hydrogen peroxides, and their production is associated with related proteins and sugar's oxidative insult. Furthermore, AGEs also produce ROS by the metal-catalysed Fenton reaction that results in site-specific attack on proteins and lipid peroxidation [233, 318-320].

Consequences of oxidative stress in AD and diabetes

Impaired insulin signaling

Some studies reported that high concentrations of H_2O_2 are able to activate insulin signaling and induce insulin-associated metabolic actions leading to increased glucose uptake, stimulation of GLUT4 translocation and lipid synthesis [321, 322]. Furthermore, under oxidative stress, stress-associated signaling cascades like MAP kinase become activated and induce phosphorylation of insulin receptors (IRS) resulting in protein degradation and the release of IRS from membrane pools [323, 324]. All these processes accumulatively result in impaired insulin signaling.

Lipids and protein oxidation

Lipids and proteins are important constituents of nutrition, and a growing body of literature points out changes in their respective metabolism in AD and diabetes. Several lipid and protein, oxidative products and their derivatives are consequences of oxidative stress in AD and diabetes, and might be able to predict the onset and progression of these diseases. Cell lipids especially cholesterol and polyunsaturated fatty acids (PUFAs) are most susceptible to oxidative stress and their oxidized derivatives are more reactive than parent compounds [325, 326]. Oxidation of cells, plasma and tissue proteins is also very prominent in AD and diabetes. Oxidative damage to the proteins promotes unfolding and conformational changes that lead to loss of protein's functions and aggregation of cross-linked protein structures [327]. Protein carbonylation, nitration, glutathionylation, lipid-protein interactions and AGEs formation are well described oxidative end products, and their accumulation results in the disruption of cellular functions and pathways leading to apoptosis and necrosis [303].

Oxysterols are important oxidation products of cholesterol in the brain and are associated with pro-inflammatory, pro-apoptotic and pro-fibrogenic effects [328, 329]. Furthermore, oxysterols are also involved in up-regulating APP and BACE1, and induction of Aβ peptides when studied in SH-SY5Y human neuroblastoma cells [330].

PUFAs are prominent constituents of membrane phospholipids and play an important role in the enzyme activities and membrane fluidity. Two forms of PUFAs; PUFA ω-3 and ω-6 are essential for brain homeostasis and decreased ω-3/ω-6 ratios are early markers of AD [331, 332]. α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) are well known types of ω-3 PUFAs while linoleic acid (LA) and arachidonic acid (AA) are important forms of ω-6 PUFAs [325]. Non-enzymatic peroxidation of PUFAs resulted in unstable peroxyl radical intermediates, which further converts to endoperoxides. Reduction of the chain after the insertion of second oxygen yielded in different isoforms of isoprostanes (IsoPs) like F2-IsoPs, F3-IsoPs and F4-IsoPs, and aldehydes such as malonildialdehyde (MDA), acrolein and hydroxyalkenals especially 4-hydroxynonenal (HNE) in AD as well as in diabetes [333, 334].

Induced levels of F2-IsoPs and F4-IsoPs have been reported in hippocampus, frontal, temporal, parietal, and occipital lobes of AD patients when compared to the normal individuals. As IsoPs increased to the same extent in mild cognitive impairment as well as in late AD, these are not considered as reliable indicators of disease progression [335, 336].

Although MDAs are popular oxidative stress markers, these are not considered as specific AD markers due to their presence in mild cognitive impairment as well as in healthy elderly persons. However, MDA was reported to induce oxidative stress by inhibiting ETC complexes I, II and V, and other respiratory enzymes such as a pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and superoxide dismutase [337, 338].

Other oxidative aldehyde product's acrolein and HNE have been detected in the brains of AD patients and are thought to be molecular mediators in AD pathogenesis [339]. Acrolein adducts are usually found in NFTs and induce tau oligomerization that results in aggregation of paired helical filaments. Furthermore, tau oligomerization is promoted during tau phosphorylation where acrolein has been found to induce tau phosphorylation [340, 341].

Most attention is given to HNE as it is highly reactive and makes bonds with amino and thiol groups of proteins [342]. These protein-bound HNE are considered as important contributors in formation of NFTs [343]. Moreover, HNE are also involved in production of short Aβ peptides which are also a source of oxidative stress in AD [344]. Furthermore, HNE also disrupts the binding of histones to DNA and increases chances of DNA oxidation in AD brain [345].

The oxidation of proteins at lysine (Lys), arginine (Arg), proline (Pro), threonine (Thr) and histidine (His) residues via peroxynitrite generates protein carbonyls and nitrile, both of which have shown an increase in NFTs, non-tangle bearing neurons and glia of AD patients [303].

Amino acids like His, Lys, Arg, Pro and Thr are major targets for carbonylation and resulted into the formation of AGEs, and products of lipid peroxidation. Protein carbonyls are generally stable compounds and used as markers to determine the degree and damage due to oxidative modifications *in vitro* and *in vivo* [346, 347].

Nitrosive stress is also reported in patients with AD. Amino acids Cys, Met, Tyr and Phe are major targets for protein nitration thus causing impaired redox cell signaling, inflammatory response and protein phosphorylation [348, 349].

Protein glutathionylation resulting in disulfide bonding of thiol with protein Cys-residue is important in redox signaling. Although it occurs under normal conditions, excessive glutathionylation due to oxidative stress may cause impairment in cell sensing and stress responses [350, 351].

Novel advanced oxidation protein products (AOPPs) that induce pro-inflammatory cytokines are also being used as reliable markers of protein oxidation in AD as well as for diabetes. These are structurally similar to AGEs and generate during oxidation with chlorinated oxidants [352-354].

Oxidation of low-density lipoproteins (LDL) is also important mechanism in AD and elevated LDL is correlated with brain Aβ levels. Furthermore, paraoxonase 1 (PON1) (that contributes as an antioxidant for LDL) has also been reported significantly low in AD patients [355, 356].

DNA oxidation

DNA is also susceptible to oxidative stress, and their oxidative damage plays an important role in aging as well as AD. Mitochondrial DNA (mtDNA) and nuclear DNA oxidation have been reported in the parietal cortex of AD patients. ROS attack on the DNA can result in strand break, sister chromosome exchange, DNA-protein cross-linking, translocation and formation of more than 20 oxidized base adducts [357-359]. DNA mutations due to base modifications can lead to impaired protein synthesis and functions. Several studies have reported the induction of oxidized base adducts such as 8-hydroxyguanine (8-OHG), 8-hydroxyadenine (8-OHA), 5-hydroxyuracil (5-OHU) and 5-hydroxycystosine (5-OHC). Furthermore, failure of 8-OHG repairs has also been reported in patients with AD [358, 360, 361].

RNA oxidation

The bases in RNA are more prone to oxidation than those in DNA as they are more exposed in the single-stranded RNA molecule and are not as well protected as DNA, which uses histones as,

dedicated packaging proteins. The non-coding RNAs are also involved in synapses, neuronal specification and differentiation, and regulation of dendritic spine development. So their damage due to oxidative stress contributes to development of neurodegenerative diseases, especially AD [362-364]. Nunomura et al. extensively reviewed the RNA oxidation in neurodegenerative diseases and had discussed the biological significance and cellular mechanisms that protect against RNA oxidation [363].

Antioxidant therapy for AD and diabetes

An effective antioxidant treatment can minimize the cellular damage and reduce the burden of oxidative insult. Antioxidant mechanism may involve the enzymatic or non-enzymatic approaches. A couple of antioxidants such as vitamins, glutathione, catalase, superoxide dismutase, α-lipoic acid, coenzyme Q_{10}, carotenoids, flavonoids, minerals (zinc, manganese, copper and selenium) and cofactors (folic acid) have been tested to reduce the after-effects of oxidative stress in AD and diabetes. Several controversial studies are available about the role of antioxidants to inhibit oxidative stress and reduced AD and diabetes incidence. Unfortunately, until date all large-scale clinical trials have failed to demonstrate any influential benefits for AD and diabetic patients [285, 365-367].

Impaired calcium homeostasis

Normal intracellular calcium homeostasis is important for neuronal functions and survival. Impaired calcium homeostasis is also a consequence of mitochondrial dysfunction and intracellular calcium levels in neurons of AD patients containing NFTs were reported higher than usual. Furthermore, neurons containing NFTs susceptible to degeneration also exhibit increased levels of calcium-dependent protease protein kinase II [368]. Calcium homeostasis also regulates transglutaminase activity. Transglutaminases catalyze the formation of covalent bond between proteins that exhibit high resistance to proteolytic degradation. Higher levels of transglutaminase enzyme in the prefrontal cortex of AD patients lead to cross linking of tau proteins that resulted in NFTs [369]. Calcium homeostasis abnormalities also lead to induce production of Aβ peptides and increased calcium levels in AD patients which are thought to be involved in enhancement of proteolytic cleavage of APP to produce Aβ [370]. Aβ formation in AD patients leads to mitochondrial permeability transition pore (mPTP) opening also induces mitochondrial calcium uptake and can in turn facilitate the Aβ formation and aggregation [137]. Abnormal calcium homeostasis is commonly observed in diabetic patients and high calcium levels in diabetic pancreatic β-cells were found to disrupt insulin secretion [371]. Furthermore, several studies have proposed the impaired calcium homeostasis in diabetic receptive neurons and have observed a steady increase in intracellular calcium levels. Moreover, depolarization of inner mitochondrial membrane was observed in sensory

neurons of STZ-diabetic rats under increased calcium flux [372, 373]. These studies confirmed that impaired calcium homeostasis is also an important reason for mitochondrial damage in diabetes and AD.

Summary of the section

AD and diabetes, both result in increased oxidative stress and impaired antioxidant systems. Although many studies suggest oxidative stress as a main cause for AD and diabetes, from the above observations, it seems that oxidative stress is a consequence of these diseases and not a cause. Oxidative stress can lead to damage in several metabolic processes, and once it starts can promote AD and diabetes, which are summarized in Figure 2.4.

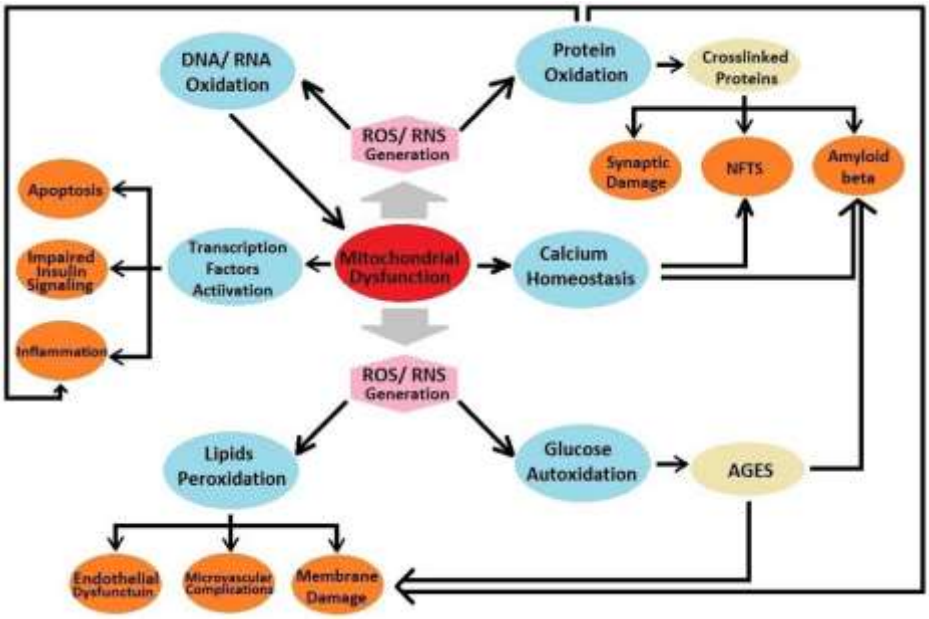

Figure 2.4: Schematic description of cellular events linked with mitochondrial dysfunctions, especially oxidative stress in diabetes and AD. Oxidative stress not only promotes inflammation and apoptosis but also foster the synaptic damage via membrane damage and enhancing production of NFTs, and Aβ. Furthermore, oxidative stress also modulates protein and lipid oxidation that resulted in microvascular complications and endothelial dysfunction in AD and diabetes.

Although several anti-oxidative stress therapies have been proposed, so far none have been useful for the treatment of AD and diabetic patients. This may be due to the involvement and overlapping of a number of mechanisms at a time such as abnormal glucose, proteins and lipid metabolism, defects in body immune system and inflammation, etc.

Common therapy for Diabetes and AD

Our above discussion on shared pathology between diabetes and AD leads to the fact that drugs used for diabetes treatment should be helpful for AD patients as rendered by other authors also. Noninsulin hypoglycemic agents such as thiazolidinedione that stimulates peroxisome proliferator-

activated receptor-γ (PPARγ) and control lipid and glucose metabolism by regulating insulin-sensitive genes and reduction in insulin resistance; are being used to treat AD, and an improvement in glucose utilization and neuronal mitochondrial biogenesis is observed in AD patients [234, 374]. A recent study by Sato T et al. has reported reduced fasting plasma insulin levels, increased insulin sensitivity and improved cognition in mild AD patients with type 2 diabetes when treated with PPARγ agonist pioglitazone. These results have shown that PPARγ agonist might be useful for pre-initial and mild AD patients as well as for AD patients with diabetes [375]. While PPARγ are also involved in regulation of APP-cleaving enzyme BACE1, PPARγ over expression leads to the formation of amyloidic proteolysis of APP and generation of Aβ plaques [376]. Whereas PPARγ agonist ginsenoside Rg1 extracted from ginseng was found to inhibit the transcription and translation of BACE1, overwhelm the BACE1 activity and eventually reduce Aβ plaques generation. Degeneration of oligodendroglia (oligodendrocytes) associated with neuronal support, and insulation is an important hallmark of AD. Recent research found that oligodendrocytes require intact insulin/IGF signaling to maintain their functions and survival. PPARγ agonist induces the MAG-1 expression in oligodendrocytes and enhances DNA repairing [377]. Current studies have shown that PPARγ agonists are also useful in reducing oxidative stress, inflammation and apoptosis in AD patients [378, 379].

Acetylcholine (ACh), a critical neurotransmitter in cognitive function is emerging possible link between AD and diabetes. Impaired insulin mechanism reduced both ACh and acetylcholine transferase (ChAT) levels, an enzyme responsible for ACh production. PPARγ agonist induced expression of ChAT that brings an improvement in learning, and memory in patients with AD [66, 380].

ACh esterase inhibitors are widely used in treatment of AD. A recent study observed that tacrine (a drug form of ACh esterase inhibitors) might reduce the diabetes induced cognitive deficits in the mice model by dysfunction of the central cholinergic system [381].

A latest study in 2012 has showed that antineoplastic agents such as bexarotene previously used for lung and breast cancer have been found to reduce Aβ plaques more than 50% within 72 hours' treatment [382]. ApoE gene expression that normally promotes the Aβ plaques generation is regulated by the ligand activated nuclear receptors PPARγ and LXRs (liver x receptors), and their binding with RXRs (retinoid X receptors) [383, 384]. Bexarotene is RXR agonist and reduced Aβ plaques in an ApoE manner by increasing its expression. We hypothesize the use of Bexarotene will be useful for diabetic patients as RXR agonists also functions as insulin sensitizers and can reduce hyperinsulinemia and hyperglycemia [385].

Metformin is a famous anti diabetic drug and has some contradictory results on AD pathogenesis. Chen et al. observed the induced Aβ formation through AMPK pathway in N2a695 cell line. This induction was inhibited through AMPK pathway pharmacological inhibitor Compound C [386]. On the other hand, metformin induced activity of PP2A and reduced tau phosphorylation in the human tau transgenic mice, and the authors suggest a beneficial role of metformin for AD therapy [387]. A recent study by Gupta et al. showed that metformin restored ACh esterase activity and inhibited GSK3β and ERK activity, and reduced Aβ production in patients with AD by sensitizing neuronal insulin resistance [388].

ABAD as described earlier has an important role in mitochondrial membrane permeability, converting estradiol to estrone. Optimal levels of estradiol are significant for neuronal survival. A current study has found that inhibition of Aβ using AG18051 restores the Aβ induced deregulation of estradiol and reduced ROS. Furthermore, when AG18051 was tested for hIAPP toxicity, partial protection was observed in type 2 diabetic pancreas. This study also pointed out a shared pathological relationship between AD and diabetes [304].

In summary, the above information lead to the assumption that AD and diabetes shared common pathology and treatment for one disease may be useful to the other due to the shared metabolic pathways.

C. elegans models of Alzheimer's disease and diabetes

A system biology approach to study the animal metabolism will benefit our understanding of diseases of old age, but the mouse, which is the traditional model for diabetes and Alzheimer's, is not the most amenable to systems biology research. In contrast, *C. elegans* may provide an excellent model for system biology of metabolism, but is not typically used as a model of these diseases. However, the use of *C. elegans* in the age related diseases like diabetes, and AD has gained greater importance in recent years due to its short lifespan and ease handling [389].

C. elegans has well established nervous system (composed of 302 neurons) having most mammalian-like neurotransmitters like serotonin, ACh, GABA, glutamine and dopamine. Almost ~42% of the human disease genes have an orthologue in *C. elegans*. Moreover, due to its simple structure, *C. elegans* provides an excellent system to study genetic manipulations based on well described genetic techniques like RNAi, etc. Furthermore, its transparent structure made it useful in fluorescent labeled neuronal studies [389, 390]. The above-described characteristics made *C. elegans* a popular lab model to study metabolic and neurodegenerative disorders.

C. elegans has been widely used to study the role of genetic and metabolic factors that modulate Aβ and tau toxicity *in vivo*. Although *C. elegans* has APP- related gene ALP, due to absence of β-secretases [391], *C. elegans* is unable to produce Aβ. To overcome this limitation, human Aβ expressing *C. elegans* models have been developed that express Aβ in muscles or neurons. The common promotors used to express Aβ in *C. elegans* muscles are *unc-54* and *myo-3*, while *snb-1* is used to express Aβ in neurons [55, 57]. Moreover, a recent *C. elegans* model express human Aβ specifically in glutamatergic neurons was successfully used to observe targets involved with cytoskeleton genes [392]. The expression of Aβ in worms is associated with time dependent paralysis, reduced lifespan and impaired behaviors.

Tau hyperphosphorylated aggregates have been known as another hall mark of AD. *C. elegans* has a tau homolog known as PTL-1 [393, 394]. Wild type and mutant human tau were expressed panneuronally in *C. elegans* using *aex-3* promoter and found to be hyper phosphorylated at several threonine and serine residues [395]. To mimic AD-relevant tau modifications, Brandit et al created a pseudohyperphosphorylated (PHP) tau model that expresses human fetal tau panneuronally [58]. Another model uses the *mec-7* promoter to express tau in the six touch neurons [396]. The worms model expressing tau have impaired movement, degeneration of motor neurons and declined touch response.

Diabetes is generally defined as metabolic disorder due to impaired glucose homeostasis. *C. elegans* offers reliable tools to study glucose homeostasis and corresponding energy metabolism [397] . Several models containing mutations at daf-2 and daf-16/ FOXO pathways are available [398, 399]. Well studied insulin signaling system provides an important tool to examine the effects of glucose- mediated damages in neurodegenerative models [400]. Moreover, usage of RNAi makes it easy to study the role of specific genes linked to energy metabolism in *C. elegans*. *C. elegans* fed with high glucose concentrations in media showed reduced lifespan, induced oxidative stress, and increased fat accumulation. Meanwhile, glucose restriction not only induced lifespan but also improved insulin sensitivity in worms [401, 402]. In addition, insertion of single gene mutations favor study the diabetes- mediated potential obesity therapeutics in *C. elegans* [403].

The formation of misfolded proteins, AGEs, mitochondrial disorders, generation of oxidative stress, insulin resistance and abnormal glucose metabolism are main hallmarks of diabetes and AD. *C. elegans* showed the presence of AGE formation, high oxidative stress and reduced lifespan when exposed to elevated glucose concentrations [reference, Schlotterer A et al]. In addition, high caloric diet also induces the Aβ formation in *C. elegans* [53]. Moreover, insulin signaling pathway present in *C. elegans* is well defined and many groups studied the genes and sub-

pathways involved in insulin signaling in *C. elegans* and found that reducing insulin signaling decreases Aβ toxicity [132]. In recent years several studies used, *C. elegans* as a model of drug research/ screening for diabetes and AD. They studied the effect of diabetic drugs on AD *C. elegans* models and vice versa. Here we briefly describe the results from these studies.

Traditional Chinese medicine, Ginkgo biloba is found to reduce insulin resistance by lowering glucose levels by significant increase and improved insulin concentration in patients with diabetes. It was also capable of reducing lipid and protein oxidation in diabetic patients [404]. Ginkgo biloba extract increases stress resistance and extend life span of *C. elegans*. Study using Ginkgo biloba leaf extracts showed reduction in Aβ induced paralysis in transgenic *C. elegans* [405]. Furthermore, increase in lifespan was observed when metformin was used in type 2 diabetic *C. elegans* model system [406]. A recent study using *C. elegans* as a model by Saharia et al. found that reserpine (FDA approved drug for AD whose mode of action and pathway's activation was unknown) acts through ACh mechanism [407]. This information lead towards the conclusion that *C. elegans* not only be used to study the effects of known drugs but may also help to elucidate the mechanisms and actions of newly developed drugs and formation of new drugs based on these results.

Future directions

The fact that some drugs developed to treat diabetes also had a positive effect on AD patients supports the view that there is a common metabolic basis for both disorders. The suggestion that AD should be referred to “type-3 diabetes” was first proposed in 2005, but the name was not widely accepted. The resurgence of metabolic research into the form of metabolomics has coincided with renewed interest in a metabolic basis of AD and there is a question mark as to whether it represents a neural form of diabetes or not.

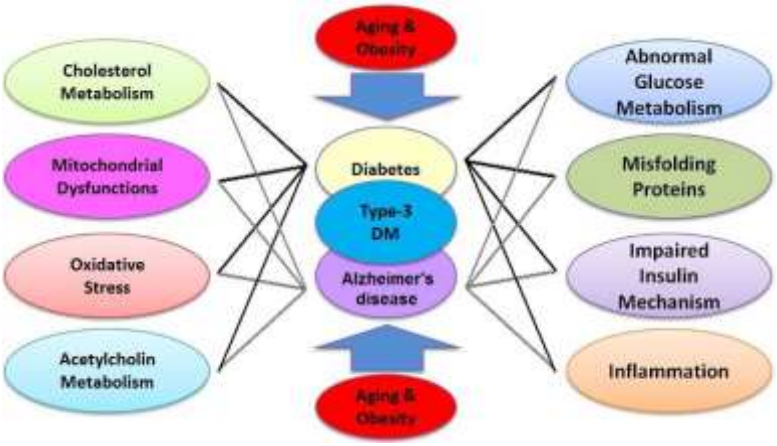

Figure 2.5: Schematic illustration of the common links between diabetes and AD. These common links lead to the hypothesis that AD may be a type of diabetes “type-3 diabetes."

The above discussion reveals that diabetes, especially T2DM, and AD follow the same pathological mechanisms resulting in misfolded proteins, insulin impairment, irregular glucose metabolism, abnormal fatty acid metabolism, mitochondrial dysfunction, and high oxidative stress. These shared metabolic profiles, and diabetes as the extreme risk factor for AD lead to the assumption that AD may reflect type-3 diabetes (Figure 2.5). Recent studies have observed the mechanistic links of the other pathways such as ApoE 4 allele, decreased ACh synthesis, PPARγ activation, and activation of inflammatory genes also shares their roles in AD and diabetes pathology. Conclusively, both AD and diabetes are resulted from metabolic abnormalities, and impaired insulin mechanism is the main reason for these defects. Future studies need deep exploration of energy metabolism associated with these mechanisms as recent studies observed direct link between lower energy metabolisms, induced lifespan, and reduced aging. Although model system *C. elegans* has some drawbacks and may not perfectly stand for the human diseases pathophysiology, it may be used as a powerful model for screening drugs, and studying diabetes and AD pathophysiology due to conserved insulin signaling and other metabolic pathways.

CHAPTER # III: Materials and Methods

Nematode strains

C. elegans strains used in this study are the wild type strain, N2 (Bristol), and the long-lived, stress resistant *dld-1* mutant, *dld-1*(*wr4*) [408, 409]. We used two strains that express the human β-amyloid peptide in muscle cells. CL2006 (dvIs2 [pCL12(*unc-54*/human Abeta peptide42 minigene) + pRF4]), which produces the human $A\beta_{42}$ peptide constitutively and CL4176 (*smg-1*(cc546) I; dvls27[pAF29(*Pmyo-3*::Aβ42) + pRF4]x), in which $A\beta_{42}$ peptide expression is increased when the temperature is increased from 16ºC to 23ºC. The use of these strains as a worm model of AD was documented previously [55, 405, 410]. The strain CL802 [*smg-1*(cc546) I;rol-6(su1006) II] was used as a control for CL2006 and CL4176 in assaying paralysis/ movement [410, 411]. We also used strain CL2355 (dvIs50 [pCL45 (*snb-1*::Abeta 1-42::3' UTR(long) + *mtl-2*::GFP] I), in which Aβ is also expressed under temperature control, though in this case the peptide is expressed pan-neuronally. The control strain for CL2355 was CL2122 (dvIs50 [(pD30.38) *unc-54* (vector) + (pCL26) *mtl-2*::GFP] I) [55, 57, 412, 413]. Sod-3::GFP reporter strain CF1553 (muIs84 [(pAD76) *sod-3p*::GFP + rol-6(su1006)]) was also used in this study. [412, 413]. Human tau expressing strain VH255 (hdEx82 [*F25B3.3*::tau352(WT) + *pha-1*(+)], genotype: *pha-1*(e2123) III; hdEx82) was also used in this study. The strain VH255 expresses fetal 352aa CNS tau in neurons [58]. Strain RB1342 [*ogt-1*(ok1474)III] was used as negative (–ve) control for O-GlcNAcylation study. Sod-3::GFP reporter strain CF1553 (muIs84 [(pAD76) sod-3p::GFP + rol-6(su1006)) was also used in this study. All the strains were obtained from the Caenorhabditis Genetics Center (CGC).

Culture conditions

Worms were maintained on nematode growth medium (NGM) seeded with *E. coli* OP50 at 20ºC, except strains CL4176 and CL2355, which were maintained at 16°C to suppress Aβ expression. Synchronized cultures for bioassays were obtained using the standard bleaching procedure [414]. Briefly, gravid hermaphrodites were exposed to a freshly prepared alkaline bleach solution (0.75N NaOH + 1.5N NaOCl). The worms were incubated in the bleach solution for five minutes at room temperature followed by centrifugation at 1100 RPM for 1 minute at room temperature. The supernatant was then discarded, and the pelleted eggs were resuspended in M9 buffer (6 g/L Na_2HPO_4; 3 g/L KH_2PO_4; 5 g/L NaCl; 0.25g/L $MgSO_4$ •$7H_2O$. Washing with M9 buffer was repeated 3 more times, after which the eggs were suspended in M9 buffer and allowed to hatch overnight on an orbital shaker. The resulting L1 larvae were shifted to the fresh NGM agar plates seeded with *E. coli* to initiate growth. Aβ inducible transgenic worms were initially cultivated at 16ºC for 36 hours after which the temperature was increased to 23ºC for 36 hours except for the

paralysis assay for which the temperature was further increased to 25ºC to maximize the effect of the Aβ transgene. Aβ constitutive expressing worms were cultured at 20ºC. To select array in VH255 strain, eggs were shifted to freshly made NGM plates and placed at 25ºC overnight. Phenotypes of the worms were monitored by visual observation under a microscope or quantified using the WormScan procedure [415].

Gene suppression by RNAi

The *E. coli* strains SJJ_LLC1.3, SJJ_T20B5.3 and SJJ_K04G7.3 (Source Bioscience), which expresses double-stranded RNA of the *dld-1, oga-1 and ogt-1* genes, respectively, were fed to the worms to suppress relative genes [416]. Briefly, the bacteria were cultured in LB medium containing 100 µg/mL ampicillin overnight with shaking at 37ºC. 300 µL of this bacterial culture was transferred to NGM plates containing 100 µg/mL ampicillin and 1 mM IPTG. The plates were incubated at 25ºC overnight to allow the bacteria to grow. Synchronized L1 worms were transferred to the bacterial plates and kept at 16ºC for 36 hours. After a further 36 hours at 20 or 25ºC, the worms were ready for use in the assays described below. Mock gene suppression controls were treated in exactly the same way except that the bacterial strain (HT115) for the controls contained the plasmid vector without specific gene fragment.

Paralysis assay

Synchronized L1 stage CL4176 worms were transferred to NGM plates. To assess the role of chemical inhibition on paralysis behaviour of Aβ transgenic strains, different concentrations of chemicals were used in normal NGM plates. Transgenic worms were exposed to these chemicals continuously - before, during and after the induction of Aβ expression. After 36 hours at 16°C, worms were shifted to 25ºC and scored for paralysis every 24 hours. Scoring began 24 hours after the temperature was raised to increase transgene expression.

Motility assay

Motility assays were performed at 20°C on synchronized L4 worms after inducing Aβ expression at 23ºC as described for *dld-1* gene suppression. Fifteen (15) animals of each strain were selected arbitrarily and put on freshly made NGM plate. Worms were left on plates for 2 minutes to allow them to equilibrate to the new conditions. Worms were next touched on the head or tail region using a platinum wire to stimulate locomotion and body bends were then counted for 30 seconds to measure movement.

Aldicarb and levamisole assays

Synchronized L1 worms were placed at 20°C on NGM plates or NGM plates supplemented with 25mM metformin that had been seeded with a lawn of OP50 *E. coli.* The worms were allowed to

grow until they reached the L4 stage of development, at which time they were shifted to new NGM plates containing 1mM aldicarb, an ACh esterase inhibitor [417] or 0.2mM levamisole, a cholinergic agonist [418]. Worms that had matured in the absence of metformin were exposed to the toxin without metformin and those exposed to metformin during maturation were, likewise, exposed to metformin during exposure to the toxin. The number of active worms was counted every half an hour until all worms became paralyzed.

Serotonin assay

To determine the level of Aβ-induced serotonin hypersensitivity, serotonin (creatinine sulfate salt) was first dissolved in M9 buffer to 1 mM as described previously [419, 420]. Synchronized worms were then washed with M9 buffer, and worms were transferred to 200 µl of the 1 mM serotonin solution in 12-well assay plates. The worms were scored as either active or paralyzed after 5 minutes.

Chemotaxis assay

The chemotaxis assay was performed as described previously [57, 421]. Worms were placed on the center of the assay plate, which was then incubated for 1 hrs at 20ºC. Attractant containing 1 µl of odorant (0.1% benzaldehyde in 100% ethanol) was added in a spot on one edge of the plate with 1 µl of 100% ethanol as a control on the opposite side of the plate. 1 µl of 1M sodium azide was added to each of the two spots to immobilize the animals once they had migrated to one or the other destination. The chemotaxis index (CI) was calculated as ([number of worms at the attractant location - number of worms at the control location]/total number of worms on the plate).

Egg viability assay

Transgenic control worm of strain CL2122 that did not express Aβ together with the Aβ transgenic strain CL2355 were synchronized as described under *culture conditions* above. After synchronization worms were grown at 16°C on NGM agar plates seeded with OP50 bacteria that either contained or did not contain 25mM metformin. Once the worms reached maturity at three days of age, 10 individuals in each of three replicate were transferred to fresh agar plates of the same composition and shifted to 23ºC. Egg viability was determined as the percentage of eggs that could hatch over the next 3 days.

Phosphine exposure assay

Nematodes were fumigated with phosphine at 500 ppm and 2000 ppm as described previously [422]. Briefly, a synchronized population of 48 hours old (L4) nematode was washed with M9 buffer and approximately 80-100 nematodes were transferred to each well of 12-well tissue culture plates containing 2.5 mL of NGM agar per well pre-seeded with either empty vector strain HT115

or E. coli strain SJJ_LLC1.3. Nematodes were exposed to phosphine for 24 hours in glass fumigation chambers. The chambers were opened, and worms were allowed to recover for 48 hrs in fresh air with lids on plates. The numbers of surviving nematodes were then counted.

Chemical inhibition of DLD-1 complex in *C. elegans*

Briefly, fresh NGM plates containing 1, 2.5, 5, 10, 25 and 50mM concentrations of 5-methoxyindole-2-carboxylic acid (MICA) were prepared. As MICA is partially soluble in water, complete solubilization was achieved by adding a drop wise 5N sodium hydroxide until MICA becomes clearly soluble in water. Paralysis assay was perfomed on worms treated with and without MICA after 36 hrs temperature up-shift at 25^{o}C.

Reversal of MICA-mediated effects

Transgenic worms were synchronized and placed at NGM plates containing *dld-1* RNAi and/ or 5mM MICA for 36 hours at 16^{o}C and afterwards up-shifted to 23^{o}C for subsequent 36 hours. L4 staged worms were next shifted to NGM plates containing different concentrations of calcium ionophore A23187 (CI) ranging from 15-45µM with and without presence of 5mM MICA and/or *dld-1* RNAi at 25^{o}C. Worms were counted for paralysis until all worms become paralyzed. To check whether absence or induction of calcium in NGM medium effects/ reverse MICA protection, transgenic worms were placed on NGM plates containing 0. 1, 2.5, 5, 7.5, and 10mM $CaCl_2$ and assessed for paralysis.

Un-coupler treatment

L4 worms were exposed to 17.5µM of the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). This dose was sufficient to stress the capacity of the worms to produce ATP without causing significant mortality of wild type nematodes. Mortality was scored immediately after a 24-hour exposure to FCCP at 23^{o}C.

Oxidative stress measurement (sod-3 expression)

Sod-3 levels were measured using sod-3::GFP reporter in CF1553 strain. Worms were fed with different chemicals for 72 hours at 20^{o}C after synchronization. Quantification of sod-3 GFP intensity level was obtained using the Zeiss fluorescence microscope. To get best signals, non-worm background fluorescence was subtracted from worm's fluorescence.

Liquid thrashing assay

Liquid thrashing assays were performed using at least 10 worms from each cohort every 2nd day until day 7. Thrashes were counted in a time interval of 10 seconds at 20^{o}C.

Glucose measurement

Synchronized day 3 (L4) worms were washed and 400µL ice cold RIPA buffer was added to each sample. Worms were sonicated, and the supernatant was collected in new tubes. Soluble protein was quantified and used as a normalizing factor during glucose quantification. This supernatant was subjected to glucose quantification by Sigma glucose assay kit (GAG*O*-20) following manufacturer guidelines.

Measurement of body size and movement

Wild type worms were synchronized and placed on normal NGM plates or plates supplemented with glucose at concentrations ranging from 0.5%-10% seeded with OP50, and placed at 20ºC. Worm body size, area and speed of movement was measured using WormLab 4.0 software (http://www.mbfbioscience.com/wormlab). Worm movements were tracked after every 24 hours for 3 days.

Western Blotting of Aβ

Aβ was identified in *C. elegans* strains by immunoblotting after separation of proteins on a 16% Tris-Tricine gel. A standard western blotting protocol was used except that SDS was omitted from the transfer buffer [423]. Briefly, synchronized L2 worms were incubated at 23ºC for 36h and then washed with distilled water, and quickly frozen in liquid nitrogen. Flash frozen worms were either stored at -80ºC or sonicated twice in the ice-cold cell lysis buffer (50 mM HEPES, pH 7.5, 6 mM $MgCl_2$, 1mM EDTA, 75 mM sucrose, 25 mM benzamide, 1 mM DTT and 1% Triton X-100 with proteinase and phosphatase inhibitors 1:100 ratio). After sonication, the lysate was centrifuged at 10000 rpm to remove insoluble debris and total protein in the supernatant was determined using the Pierce Coomassie (Bradford) protein assay kit (Thermo Scientific) measured on a Nanodrop spectrophotometer. From each sample, ~100 µg of total protein was precipitated with acetone and dissolved in Novex® Tricine SDS sample buffer (LC1676, Invitrogen) by heating to 99°C for 5 minutes. Samples were subjected to gel electrophoresis at 100V for 2.5 hrs using separate cathode (100mM Tris, 100mM Tricine, 0.1% SDS, pH 8.3) and anode (0.2M Tris, pH 8.8) running buffers. Proteins were wet transferred onto nitrocellulose membranes by electroblotting in the transfer buffer (35 mM glycine, 48 mM Tris (pH = 8.8) and 20% methanol) for 70 min at 100V and stained with Ponceau S (0.1% Ponceau S in 1% acetic acid) for 5 minutes following de-staining with 10% acetic acid (5 minutes) and washing under water (5 minutes). The membranes were blocked overnight in 5% skim milk at 4ºC to prevent non-specific binding of antibodies. The primary antibody binding was done using the mouse Aβ monoclonal antibody 6E10 (Covance) at 1:1000 dilution in TBS (50mM Tris, 150mM NaCl, pH 7.6) containing 1% skim milk for 3-4 hours at room temperature followed by three washes with TBS-T of five minutes each. Rabbit anti-actin antibody (2Q1055,

Abcam) was used to detect actin as a reference protein. Anti-mouse IgG alkaline phosphatase antibody was produced in goat (A3562, Sigma), and anti-rabbit IgG alkaline phosphatase antibody was produced in goat (A3687, Sigma) was used as a secondary antibody at 1:10000 dilutions in TBS containing 1% skim milk. Secondary antibody binding was carried out for 1 hour at room temperature. After washing the membrane three times with TBS-T at five minutes per wash, the proteins were detected using the BCIP/ NBT substrate system (Sigma) in 1M Tris (pH 9.0).

For DLD-1 detection, anti-lipoamide dehydrogenase antibody (ab133551) was used. Same procedure was repeated for DLD-1 detection except 5%BSA in 1X TBST was used for anti-body detection. Anti-mouse IgG alkaline phosphatase antibody produced in goat (A3562, Sigma), and anti-rabbit IgG alkaline phosphatase antibody produced in goat (A3687, Sigma were used as secondary antibody at 1:10000 dilutions in TBS containing 1% skim milk or in 1% BSA in 1X TBST. Secondary antibody staining was done for 1 hour at room temperature. After washing the membrane with TBST, the proteins were detected using BCIP/ NBT substrate system (Sigma) or BCIP/ NBT kit (002209) from Lifetechnologies dissolved in 1M Tris (pH 9.0).

For tau phosphorylation detection, synchronized worms were incubated at 20^{o}C and L4 worms (day 3) were collected and washed three-four times to remove the bacterial traces. Half of the washed worms were added in 400µl cold RIPA buffer containing proteinases and phosphatases inhibitors. The remaining half of the worms were shifted to new plates containing 75µM FUDR to restrict progeny production and collected on day 7. Worms were subjected to sonication, and lysate was collected for further protein analysis. Protein was measured and about 50-80µg protein was subjected to 10% SDS-PAGE gels for two hours. Proteins were transferred to nitrocellulose membranes for further analysis in SDS- containing transfer buffer. For Aβ, the membranes were blocked overnight in 5% skim milk at 4^{o}C to prevent non-specific binding of antibodies. For tau protein extracts; membranes were blocked in 5% BSA in 0.1% TBST ((TBS containing 0.1% Tween 20). Primary antibody staining was done using the Aβ monoclonal antibody 6E10 (Covance) at 1:1000 dilution in TBS (50mM Tris, 150mM NaCl, pH 7.6) containing 1% skim milk for 3-4 hours at room temperature following three washes with TBS-T five minutes each. A number of antibodies were used to assess total tau and site-specific phosphorylated tau. The antibodies used for tau were, Tau-5 (ab80579, Abcam), HT7 (MN1000, Thermo Scientific), phosphor S198 (ab79540, Abcam), phosphor S235 (NB100-82241, Novus Biologicals), phosphor S262 (79-152, Prosci), phosphor S396 (710298, Novex Lifetecnologies), AT8 (S202/ Thr-205, MN1020, Thermo Scientific), and AT180 (T231/ Ser-235, MN1040, Thermo Scientific). For O-GlcNAc detection, anti-O-GlcNAc antibody (CTD110.6, sc-59623, SantaCruz) was used. Anti-actin antibody (2Q1055, Abcam) or anti-γ-Tubulin (T1450, Sigma) were used as reference control. Anti-mouse

IgG alkaline phosphatase antibody produced in goat (A3562, Sigma), and anti-rabbit IgG alkaline phosphatase antibody produced in goat (A3687, Sigma was used as secondary antibody at 1:10000 dilutions in TBS containing 1% skim milk or 1% BSA in 1%TBST. Secondary antibody staining was done for one hour at room temperature. After washing the membrane with 1X TBS-T, the proteins were detected using BCIP/ NBT substrate system (Sigma) or BCIP/ NBT kit (002209) from Lifetechnologies dissolved in 1M Tris (pH 9.0).

Quantitative RT-PCR and immunoblotting confirmation

Aβ mRNA levels in transgenic worms either exposed or not exposed to 25mM metformin were quantified using Rotor-Gene Q (QIAGEN) thermocycler. Briefly, total RNA was extracted using the acid-phenol (Trizol) method and converted to single-stranded cDNA using Invitrogen SuperScript cDNA synthesis kit following the prescribed protocol. Gene-specific primers were as follow: Aβ forward primer CCGACATGACTCAGGATATGAAGT, Aβ reverse primer CACCATGAGTCCAATGATTGCA; *dld-1* forward primer GATGCCGATCTCGTCGTTAT, *dld-1* reverse primer TGTGCAGTCGATTCCTCTTG; *act-1* forward primer CGCTCTTGCCCCATCGTAAG, *act-1* reverse primer CTGTTGGAAGGTGGAGAGGG; *gapdh-2* forward primer TTCTCGTGGTTGACTCCGAC, and *gapdh-2* reverse primer AGGGAGGAGCCAAGAAGGTAAC. The PCR conditions were 95ºC for 30 s followed 35 cycles of 95ºC for 20 s, 55ºC for 30 s, and 72ºC for 40 s. Following amplification, a melting curve was determined during a temperature ramp from 72ºC to 95°C. For qPCR quantification, SYBR® Green JumpStart™ ReadyMix™ (Sigma) was used.

Protein levels were also quantified by immunoblotting for comparison. A simple procedure was used to transfer whole-cell lysate proteins on nitrocellulose membrane. Briefly, equal amounts of each protein sample were subjected to SDS gel electrophoresis using only a stacking gel. Once the proteins were run through the gel, they were transferred to a nitrocellulose membrane using a standard protocol. Transferred proteins were incubated either with anti-Aβ primary antibody 6E10 or anti-actin antibody (2Q1055, Abcam).

Statistical analysis

Paralysis curves were compared using log-rank (Mantel-Cox) test. Pairwise treatments were analyzed for statistical significance by independent student's *t*-test using GraphPad prism 6.0d. Log-rank estimate was applied in survival calculations. A *p* value less than 0.05 was considered statistically significant.

CHAPTER # IV: Suppression of the dihydrolipoamide dehydrogenase gene (*dld-1*) protects against the toxicity of human amyloid beta in *C. elegans*

Abstract

Declines in energy metabolism and associated mitochondrial enzymes are linked to the progression of Alzheimer's disease (AD). Dihydrolipoamide dehydrogenase (*dld*) and two of its enzyme complexes namely, pyruvate dehydrogenase and α- ketoglutarate dehydrogenase are associated with AD and have a significant role in energy metabolism. Interestingly, *dld* gene variants are genetically linked to late-onset AD; and reduced activity of DLD-containing enzyme complexes has been observed in AD patients. To understand how energy metabolism influences AD progression, we suppressed the *dld-1* gene in *C. elegans* expressing the human Aβ peptide. *dld-1* gene suppression improved many aspects of vitality and function directly affected by Aβ pathology in *C. elegans*. This includes protection against paralysis, improved fecundity and improved egg hatching rates. Suppression of the *dld-1* gene restores normal sensitivity to aldicarb, levamisole and serotonin, and improves chemotaxis. Suppression of *dld-1* does not decrease levels of the Aβ peptide, but does reduce the formation of toxic Aβ oligomers. The mitochondrial uncoupler, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) acts synergistically with Aβ to overcome the protective effect of *dld-1* gene suppression. Another metabolic toxin, phosphine, acted additively with Aβ. Our work supports the hypothesis that lowering energy metabolism may protect against Aβ pathogenicity, but that this may increase susceptibility to other metabolic disturbances.

Introduction

One of the main pathological hallmarks of AD that underlies the neuronal dysfunction and dementia is extracellular accumulation of amyloid beta (Aβ) plaques resulting from protein misfolding [48]. In addition to the accumulation of Aβ, neuroimaging studies of AD brains found induced aerobic glycolysis in areas severely affected by the disease at the preclinical stage, but reduced glucose metabolism and diminished activities of mitochondrial enzymes at latter stages of the disease [424-427]. The cause and effect relationships between these observations is unclear as impairment of energy metabolism may induce protein misfolding, leading to formation of Aβ plaques, but the opposite may also be true as production and accumulation of Aβ may also damage energy metabolism [164, 428-433].

The decrease in energy metabolism with age has been interpreted in two opposing ways; as a main cause of AD, or as a protective response against the symptoms of the disease. The first

interpretation is mostly supported by studies conducted at a late stage of the disease on post-mortem brains, making it difficult to assign causality [205, 434-437]. In contrast, recent studies on mixed stage AD samples support that the down-regulation of energy metabolism is a protective factor, leading to the hypothesis that a decrease in nutrient and oxygen supply minimizes neural activity, thereby decreasing the repair burden [45, 438]. This is supported by results from a transgenic mouse model of AD in which upregulation of aerobic respiration is clearly harmful [439].

The difficulty in understanding the role of metabolic decline on AD relates to the inaccessibility of AD affected brains during progression of the disease. This situation makes it difficult to distinguish cause from consequence and necessitates reliance on AD disease models. The presence of Aβ in mice and neuronal cell lines causes mitochondrial dysfunction by inducing mitochondrial DNA damage, disrupting mitochondrial redox potential and inducing oxidative stress [29, 440-442]. Exposure to 2-deoxy-d-glucose (2DOG), which is an analogue of glucose that inhibits both glycolysis and oxidative phosphorylation, causes a decrease in Aβ toxicity [31, 32]. Both of these results could be interpreted as lower rates of glucose metabolism having a protective effect. Furthermore, induction of oxidative phosphorylation with dichloroacetic acid (an activator of pyruvate dehydrogenase (PDH)) increases neuronal sensitivity toward Aβ [33]. These findings reinforce the interpretation that there is a positive correlation between the rate of energy metabolism and Aβ toxicity.

Increased risk of late-onset AD is genetically linked to the human *dld* locus [34]. The DLD enzyme is a subunit of three ketoacid dehydrogenase complexes, each of which contributes to energy metabolism, pyruvate dehydrogenase PDH, α-ketoglutarate dehydrogenase complex (KGDH) and branched chain ketoacid dehydrogenase complex (BCKDH) [35, 443]. A decrease in the activity of PDH and KGDH is associated with neurodegeneration as reduced activities of these complexes are observed in post-mortem brain tissues and fibroblasts of patients with either Alzheimer's or Parkinson's disease [36-40, 444]. Repressed activity of the KGDH complex, in particular by creating mice that are heterozygous for a knockout mutation of one of its subunits (E2), decreases glucose utilization in the cortex, which mimics the situation in cortical neurodegenerative diseases [41, 42]. Targeted disruption of DLD can also reduce the activities of KGDH and PDH in mice [445], which in the case of PDH blocks the connection between glycolysis and the TCA cycle. [446, 447]. Thus, a direct link between DLD activity and AD progression is a distinct possibility.

To explore the relationship between metabolism and AD, we suppressed *dld-1* in the nematode *C. elegans* that express human Aβ. *C. elegans* is well-suited for such studies as it has

been used extensively to study the genetics of aging and associated age-related diseases such as AD. Well-developed models exist in which human Aβ is expressed in either muscle or neuronal cells of *C. elegans* [57, 448]. These strains can be used to monitor the influence of metabolic changes on the aggregation of Aβ peptide either directly or through altered behaviour resulting from cellular malfunction. Assays that have been developed include progressive paralysis, a decrease in chemotactic ability and altered response to neurotransmitters. These assays allow convenient visual monitoring of the molecular processes leading up to Aβ aggregation. A decrease in Aβ mediated pathology in response to suppression of *dld-1* supports the notion that decreased energy metabolism is neuroprotective.

Results

The effect of metabolic rate on Alzheimer's disease is an unresolved issue. While a decline in respiration rate is associated with both age of onset and the severity of AD, there are possible alternative explanations. It may either trigger the age-related increase in AD, or it may be a response that protects against the progression of AD. We use gene suppression of *dld-1* to directly test the effect of metabolic suppression in several different *C. elegans* models of Aβ pathology. Specifically, we test the effect of *dld-1* gene suppression on nematodes that express Aβ either constitutively or with temperature induction in muscle cells or constitutively throughout the nervous system. The general experimental paradigm is to expose the nematodes to conditions known to result in Aβ toxicity and to determine whether genetic suppression of *dld* activity influences that toxicity. In our study *dld-1* RNAi effectively suppress the *dld-1* mRNA (Supplementary Figure S4.1).

4.1. dld-1 suppression alleviates Aβ pathology in transgenic C. elegans

Transgenic expression and deposition of Aβ in body wall muscle cells of *C. elegans* causes severe, age-progressive paralysis. (Figure 4.1A). A temperature shift to 25°C was used to induce high level expression of Aβ. Fewer than 10% of the nematodes of the CL802 control strain that lacks the human Aβ transgene were paralyzed, i.e. unresponsive to prodding by 38 hours. In contrast, 100% of the worms of the CL4176 strain that does express human Aβ were unresponsive (Figure 4.1A). Suppression of *dld-1* in CL4176 reduced the frequency of paralysis due to Aβ expression to only ~30%, whereas suppression of the *dld-1* gene did not alter the robust activity of the control strain, CL802. When we extended the time of the assay (Figure 4.1B), we found that CL4176 worms in which *dld-1* gene expression had been suppressed did not become completely paralyzed until 144±24 hours. We repeated the test on CL2006 worms in which Aβ is expressed constitutively and found that suppression of the *dld-1* gene also delayed paralysis in these worms

(Supplementary Figure S4.2). Thus, *dld-1* gene suppression prevents, to a large degree, the pathology associated with Aβ that causes paralysis.

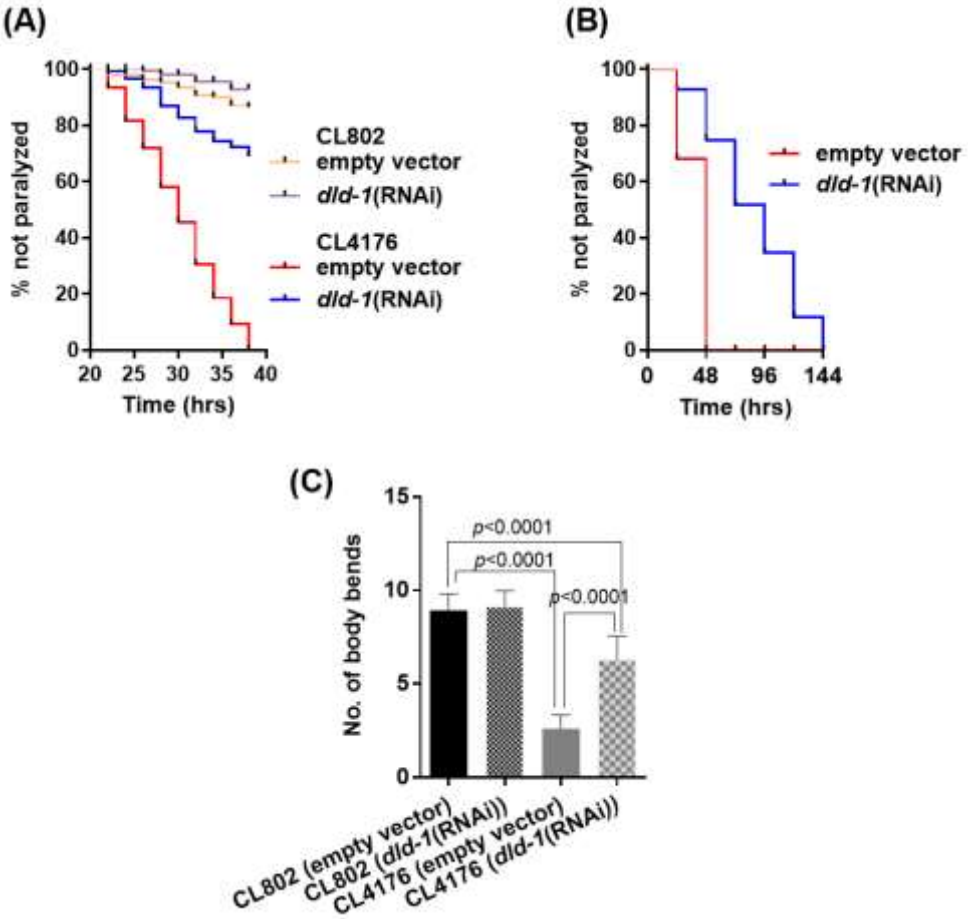

Figure 4.1: Suppression of the *dld-1* gene alleviates paralysis due to human Aβ expressed in transgenic *C. elegans*. (A) Time-dependent paralysis of the Aβ-expressing strain CL4176, with and without *dld-1* RNAi. Paralysis of synchronized L1 worms was measured on NGM plates seeded with *E. coli* strain HT115 containing either empty vector or a *dld-1* RNAi construct. After 36 hours at 16ºC, the temperature was up-shifted to 25ºC. Paralyzed worms were counted 24 hours after the temperature shift and thereafter, every two hours. (B) Extended time-dependent paralysis analysis. As *dld-1* RNAi significantly delayed paralysis, we repeated the analysis in a, but monitored the worms every 24 hours until the last worm become paralyzed. Kaplan Meyer survival curves were compared using a Log-rank test. (C) CL4176 worms were synchronized and placed on plates either seeded with *E. coli* strain HT115 containing either empty vector or a *dld-1* RNAi construct. After 36-hour incubation at 16ºC, the temperature was increased to 23ºC for 36 hours. Worms were collected, washed and transferred to fresh plates. Worms were touched at the head region with a platinum wire and total number of body bends were counted under the microscope at 20ºC. Results represented data from three independent trials (n=40-60 worms/ trial). Bars = mean ± SD.

A second movement assay was performed that involved tapping the worms with a platinum wire and counting the number of body bends for 30 seconds. The worms were prepared as for the preceding assay except that the assay was carried out at room temperature (20°C) immediately after

temperature induction of Aβ expression at 23°C. We found that as with the immobility assay, expression of human Aβ in the CL4176 strain resulted in a decrease in the rate of movement. Suppression of the *dld-1* gene by RNAi significantly improved mobility of CL4176 worms expressing human Aβ, resulting in 6.2±1.3 rather than 2.6±0.7 body bends ($p<0.0001$) (Figure 4.1C). The control CL802 worms that did not contain the Aβ transgene were unaffected by *dld-1* suppression (9.1±0.8 rather than 8.9±0.9 body bends) ($p=0.529$).

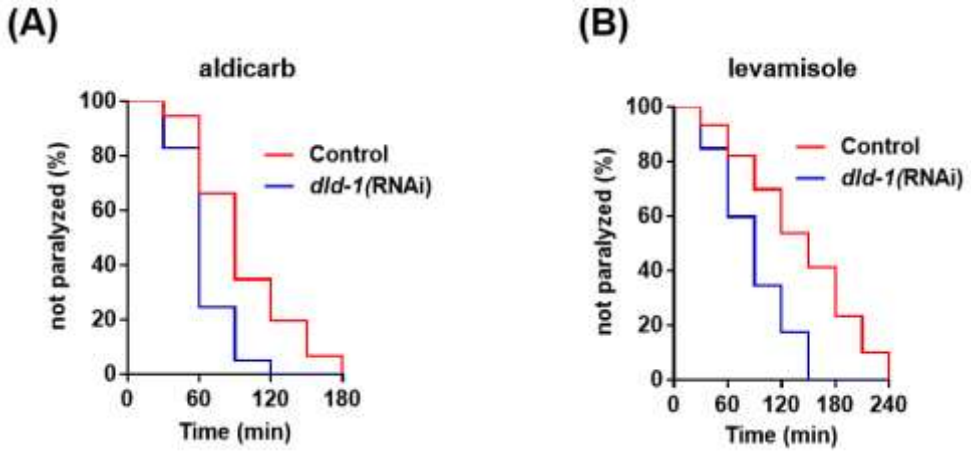

Figure 4.2: Acetylcholine neurotransmission assay in worms that express Aβ in muscle. Paralysis assay show that *dld-1* gene suppression improves acetylcholine neurotransmission in constitutive Aβ expressing *C. elegans* strain CL2006. (A) Time-dependent paralysis of control and transgenic worms fed on aldicarb (1 mM) with and without *dld-1* RNAi. (B) Time-dependent paralysis of worms fed on levamisole (0.2 mM) with and without *dld-1* RNAi. Results represented data from three independent trials (n=40-60 worms/ trial). Assay curves were compared using Log-rank test. Results represent the average of three independent trials.

Expression of Aβ in muscle cells inhibits acetylcholine (ACh) neurotransmission, which is related to the observation that ACh agonists are commonly used to delay the symptoms of Alzheimer's disease [449]. The inhibition of cholinergic neurotransmission by Aβ can be conveniently assayed by the protection it provides against a normally toxic dose of cholinergic agonist. Thus, restoration of normal sensitivity to the agonist is an indication of a decrease in the neurotoxic effects of Aβ.

To check whether *dld-1* inhibition restores normal ACh neurotransmission in CL2006 worms that constitutively express Aβ in muscle, we monitored paralysis in response to the cholinergic agonists, aldicarb (a potent acetylcholinesterase inhibitor) and levamisole (a cholinergic receptor agonist).

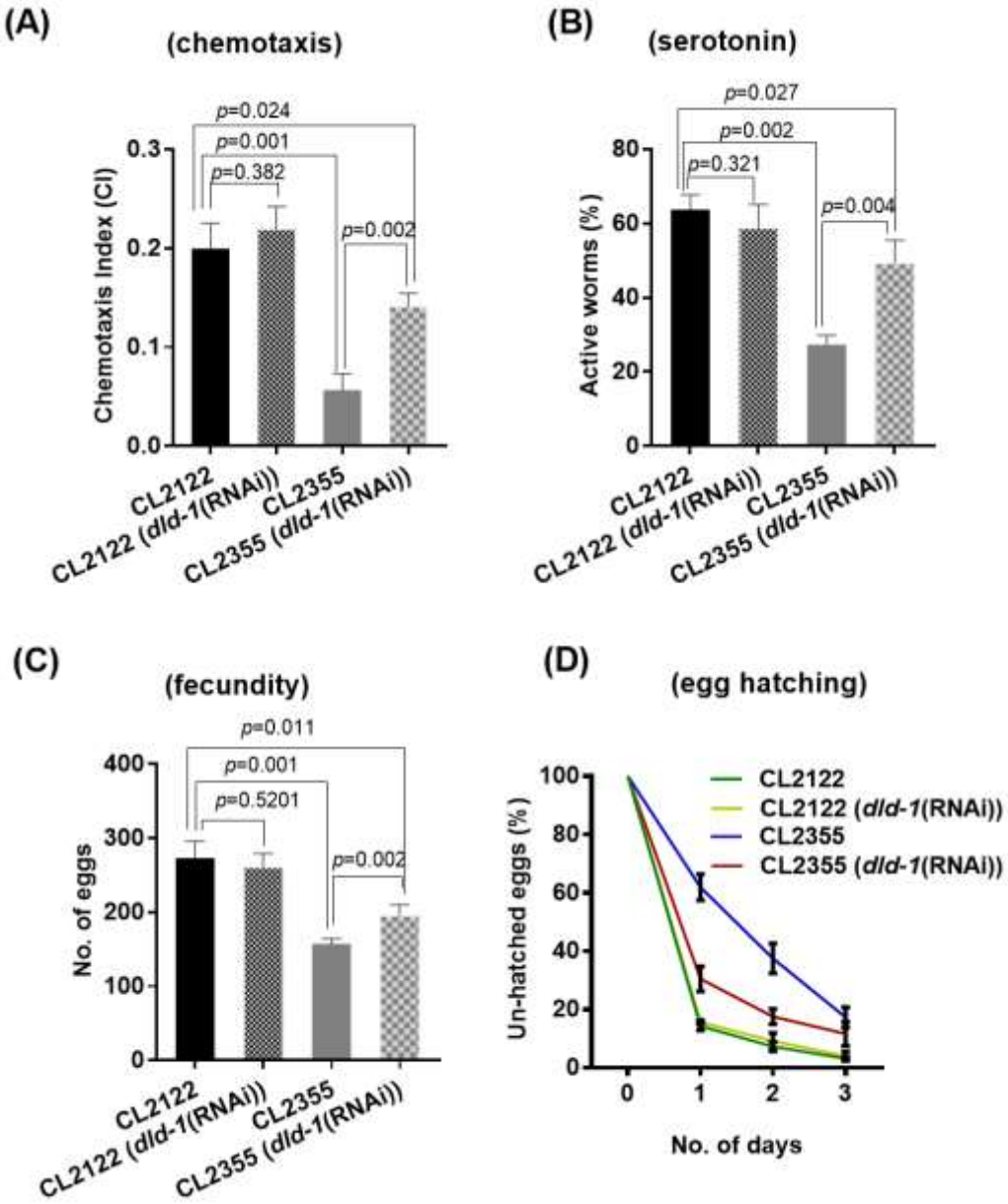

Figure 4.3: Effect of *dld-1* suppression on impaired behaviour in *C. elegans* that express Aβ in neurons. Chemotaxis, 5-HT serotonin sensitivity and, egg laying and hatching were compared between a no-Aβ control (CL2122) and that express Aβ in neurons (CL2355). Synchronized worms were fed with *E. coli* containing either empty vector or a vector that expresses *dld-1* dsRNA. (A) Analysis of chemotaxis behaviour in worms that express Aβ in neurons. Synchronized worms were placed at 16ºC for 36 hours, and then shifted to 25ºC. L4 worms were collected and assayed for chemotaxis towards benzaldehyde at room temperature (n = 40-50 worms in each well/ trial). (B) Evaluation of serotonin sensitivity in worms that express Aβ in neurons. Synchronized L4 stage worms were assessed for serotonin hypersensitivity at room temperature by placing them in a 96 well plate containing 250 µl of 1 mM serotonin (n = 25-30 worms / trial) and counted for paralysis after 5 minutes. Three independent trials were run for each experiment. (C) Fecundity of worms that express Aβ in neurons with or without *dld-1* RNAi. (D) Time course of egg hatching percentage in worms that express Aβ in neurons. For Figure 3C and 3D, worms were synchronized and placed at NGM plates with or without *dld-1* RNAi at 16ºC until L4 stage appeared. Ten L4 stage worms were picked to fresh plates at 23ºC to induce transgene expression. After 24 hours at 23°C, adults were removed. Plates

were shifted to 20ºC for remaining assay and eggs and larvae were counted each day for 3 days. Total number of eggs were estimated by adding the total number of un-hatched eggs and larvae present. Three independent trials were run for each experiment. Bars = mean ± SD.

Resistance of the CL2006 strain to ACh agonists is due to production and deposition of both Aβ oligomers and fibrils [407]. Exposure to aldicarb (Figure 4.2A) results in paralysis within 180 min, which, as expected, occurs more rapidly under *dld-1* gene suppression (within 120 min, p=0.0001). Similarly, paralysis in response to levamisole is decreased from 240 min to 150 min (Figure 4.2B) when the *dld-1* gene is suppressed by RNAi (p=0.0001). Unlike the response in strains in which Aβ is expressed, suppression of *dld-1* had no effect on the response to either aldicarb or levamisole in either wild type or *dld-1* mutant worms (Supplementary Figure S4.3). Both non transgenic strains became paralyzed earlier than transgenic worms that express Aβ (~120 min for both aldicarb and levamisole). Our results indicate that *dld-1* suppression restores near normal ACh neurotransmission in Aβ expressing worms via a decrease in Aβ-toxicity.

Additional assays have been developed to monitor toxicity of *Aβ expressed in neurons,* impaired chemotaxis, hypersensitivity toward serotonin (5-HT), and reduced fecundity and egg hatching [57, 450]. Neural expression of Aβ in strain CL2355 significantly impaired chemotaxis toward benzaldehyde (chemotaxis index=0.05±0.01) relative to the non-Aβ control strain CL2122 (CI=0.20±0.02, p=0.002) (Figure 4.3A). Whereas suppression of the *dld-1* gene did not affect chemotaxis of the control strain (CI=0.22±0.02, p=0.4), it significantly improved chemotaxis of strain CL2355 (CI=0.14±0.01, p=0.002). Suppression of the *dld-1* gene in Aβ expressing worms was unable to fully restore chemotaxis to control levels (p=0.02).

Serotonin is an important biogenic amine neurotransmitter that mediates locomotion, egg laying and feeding behaviour in *C. elegans*. Exogenously applied serotonin causes paralysis in worms, which is exacerbated by expression of human Aβ [57]. In our study, 63.6±4.1% of worms of the control strain CL2122 were active, but this was reduced to 27.3±2.6% in the CL2355 strain that expresses Aβ throughout the nervous system (p=0.002). Suppression of the *dld-1* gene did not affect the activity of the control strain CL2122, (58.6±6.5%, p=0.3), but could partially alleviate serotonin induced paralysis in CL2355, increasing the percentage of worms that were active to (49.3±6.1%, p=0.004) but not to the level of the no-Aβ control strain (p=0.03). (Figure 4.3B).

Serotonin and ACh neurotransmission control egg laying [451], an activity that is inhibited by neuronal expression of Aβ. Based on our findings above, we reasoned that suppression of the *dld-1* gene would reverse the negative effect of Aβ expression on fecundity. Aβ expression significantly reduced egg laying in CL2355 relative to the control strain, CL2122 (157±8 vs

273±23, p=0.001). While there was no significant effect of *dld-1* gene suppression by RNAi on the strain that did not express Aβ (273±23 vs 261±19, p=0.5), suppression of the *dld-1* gene caused a marked improvement in fecundity in CL2355 (157±8 vs 207±11, p=0.002). The improvement in fecundity did not reach that of the matched control (p=0.01) (Figure 4.3C).

Aβ expression also negatively affects egg hatching, with 61.8% of CL2355 eggs remaining un-hatched after 24 hours. In contrast, only 14.2% of CL2122 eggs remained unhatched. Inhibition of *dld-1* resulted in a significant decrease in unhatched eggs after 24 hours, 30.5% (Figure 4.3D). The same trend persisted over the next two days. There was no effect of *dld-1* suppression on egg hatching of the control strain CL2122.

4.2. dld-1 suppression reduces Aβ protein oligomerization without affecting Aβ peptide levels

A reduction in Aβ toxicity in our study could result from either a decrease in overall *Aβ* peptide levels or a decrease in the formation of toxic oligomers [452]. We did not observe any significant change in *Aβ* mRNA levels after *dld-1* suppression, indicating that gene expression was not affected (Figure 4.4A). We then assessed whether *dld-1* suppression affected either the total amount of *Aβ* peptide produced or the degree of *Aβ* oligomerization. We found no change in the overall level of Aβ peptide due to *dld-1* suppression (Figure 4.4 B and C). However, there was a significant decrease in the proportion of Aβ peptide in the form of ~19kDa oligomers and a corresponding increase in ~4kDa monomers (0.31±0.13 vs 0.71±0.023, p=0.045) when the *dld-1* gene was suppressed in strain CL4176 (Figure 4B and 4D). In contrast, there was no significant change in oligomers of 12kDa, 16kDa or 23kDa.

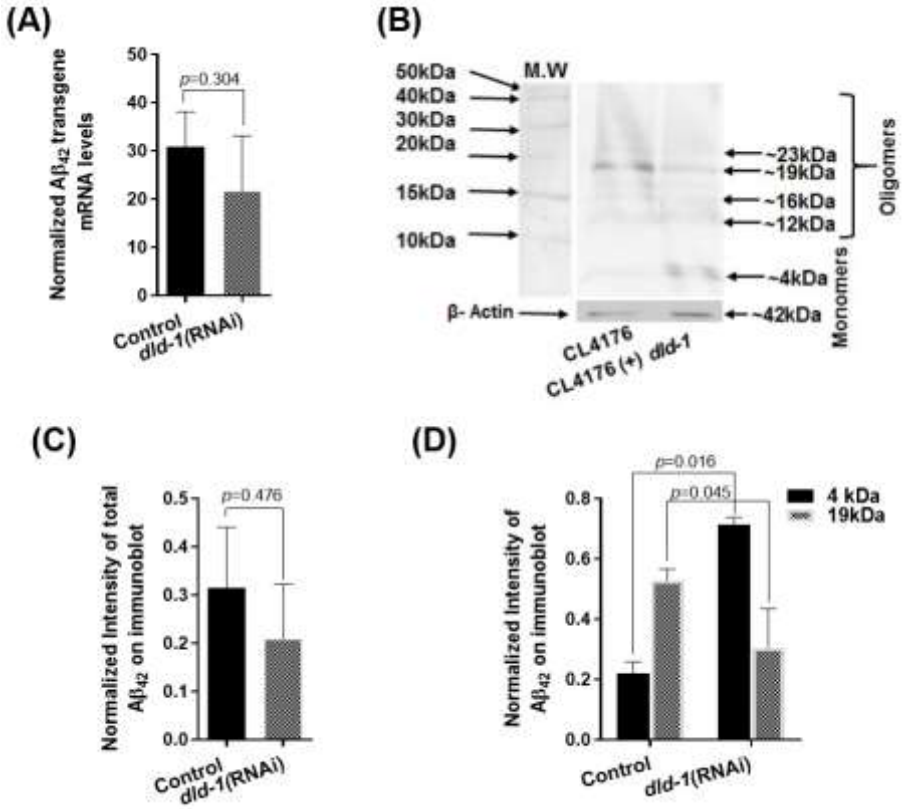

Figure 4.4: Effect of *dld-1* suppression on *Aβ* transgene and protein expression. (A) Quantitative RT-PCR of Aβ mRNA levels Synchronized worms were fed with *E. coli* containing either empty vector or a vector that expresses *dld-1* dsRNA. Worms were incubated for 36 hrs at 16ºC and then temperature was shifted to 23ºC for further 36 hrs before worms were collected for RNA or protein extraction. Levels of *Aβ* mRNA are normalized to glyceraldehyde-3-phosphate dehydrogenase (*gpd-2*) transcript levels, with experiments replicated three times. (B) A western blot of total soluble protein run on a 16% Tris-tricine gel shows multimers of Aβ *in C. elegans* strains expressing human Aβ. Arrows indicate the presence of *Aβ* monomers at 4kDa, and oligomers at 12kDa, 16kDa, 19kDa and 23kDa. (+) *dld-1* indicates treatments in which the *dld-1* gene has been suppressed by RNAi. The control using an actin antibody is shown below to indicate the relative amount of protein loaded onto each lane. (C) Densitometry of overall protein bands appeared on western blot of each column to estimate differences in Aβ protein expression after *dld-1* suppression. (D) Quantification of the intensity of 4kDa monomer and 19kDa oligomer bands with and without *dld-1* suppression from three independent trials. Quantification was carried out using GelQuantNET software. Bars= mean±SD.

4.3. How does dld-1 gene suppression protect against the toxicity of Aβ?

DLD-1 is a core enzyme of oxidative respiration and the *dld-1*(*wr4*) mutation is known to inhibit energy metabolism (Zuryn et al., 2008). FCCP is also a disruptor of mitochondrial energy metabolism, but it acts in quite a different manner. Both likely disrupt the generation of ATP, but by opposite mechanisms. DLD-1 disruption slows the flow of metabolites through the TCA cycle and the electron transport chain, whereas FCCP accelerates the same flows, but in a futile manner. Exposure to FCCP decreases Aβ production[453, 454], which implies that ATP depletion is more important to the protection than is the mechanism that causes the decrease in ATP.

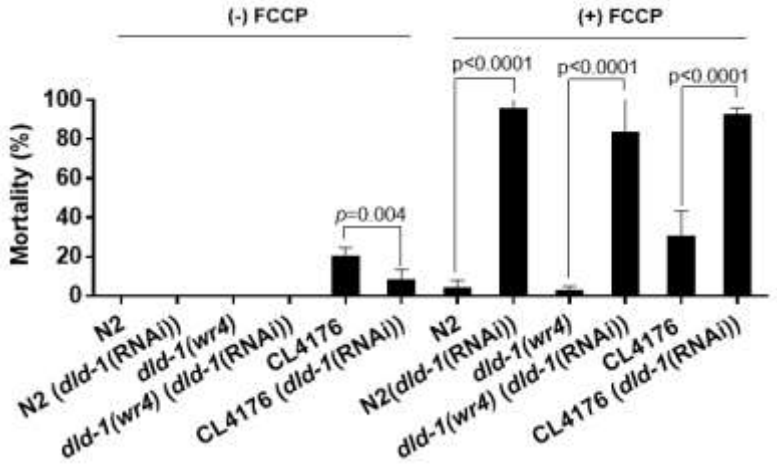

Figure 4.5: Mortality assay of *C. elegans* treated with the mitochondrial uncoupler FCCP. When the *dld-1* gene was suppressed in the presence of uncoupler, the compounded disruption of energy metabolism resulted in high level mortality in each of the three strains regardless of the presence of the *Aβ* peptide (n= 50-100 for each experiment. Three independent trials were run. Bars= mean ± SD.

To determine which of these two options, metabolite flux or ATP generation is more likely responsible for the protection against Aβ-induced toxicity, we measured Aβ-mediated toxicity in combination with either *dld-1* gene suppression or FCCP exposure or both. In the absence of FCCP, both wild type and *dld-1* mutant strains exhibited 100% survival whether or not *dld-1* was suppressed. The Aβ transgenic worms exhibited moderate mortality that was significantly alleviated by RNAi suppression of *dld-1* (20.5±4.2 vs 8.2±5.3, p=0.004).

Exposure to FCCP had an effect very different to that of *dld-1* gene suppression. The dose of FCCP that was used caused negligible mortality on its own, but rather than protect against Aβ-mediated mortality, it produced an apparent, but not quite significant increase in mortality from 20.5%±4.2 to 30.8%±12.3 (p=0.107). This outcome does not allow us to attribute protection against Aβ to a decrease in ATP generation. Mortality due to Aβ expression combined with FCCP increased greatly when *dld-1* gene expression was suppressed by RNAi (30.8±12.3 vs 92.9±2.8, p<0.0001). Mortality was not restricted to the transgenic strain, however, but also was observed in wild-type N2 (4.2±3.5 vs 95.7±4.1, p<0.0001) and the *dld-1*(*wr4*) mutant (3.7±3.3 vs 83.2±16.8, p<0.0001). The most likely explanation is that the decrease in metabolite flux due to a decrease in DLD-1 containing metabolic complexes, together with futile pumping of protons across the inner mitochondrial membrane caused by FCCP, results in a crisis of energy metabolism that affects all three strains equivalently, and that this is largely independent of whether Aβ peptide is expressed.

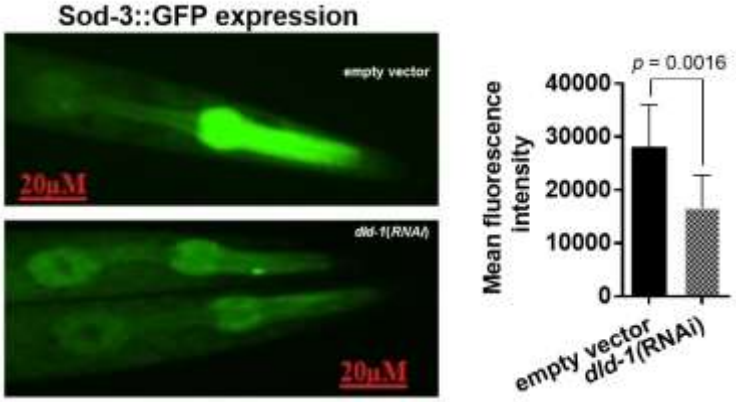

Figure 4.6: Suppression of the *dld-1* gene lowers the response to superoxide in *C. elegans.* Synchronized L1 worms of strain CF1553 that expresses *sod-3*::gfp was fed *E. coli* containing either empty vector (strain HT115) or vector that expressed *dld-1* ds-RNA for 72 hours at 20ºC. GFP fluorescence was quantified in at least ten worms from each group. Mean fluorescence intensity was measured using the formula; Integrated density – (area of selected worm × mean fluorescence of background readings). Bars = mean ± SD.

Another possible mechanism whereby *dld-1* gene suppression could reduce the toxicity of Aβ is via a decrease in reactive oxygen species (ROS), as ROS can induce aggregation of Aβ [317, 442, 455]. DLD itself can generate significant amounts of ROS (superoxide), so suppression of DLD activity could lead to a decrease in superoxide production [297, 456]. The superoxide dismutase-3 enzyme (SOD-3) converts superoxide into O_2 and H_2O_2. Because the *sod-3* gene is induced by its substrate, superoxide, we used a strain of *C. elegans* (CF1553) that expresses GFP under the control of the *sod-3* promoter to measure the effect of *dld-1* suppression on intracellular superoxide levels. Suppression of the *dld-1* gene resulted in a decrease in GFP signal (28120.3±7884.3 vs 16662.8±6145.7, p=0.0016). This indicates that suppression of *dld-1* gene expression causes a decrease in ROS, provide another plausible mechanism to explain the protective effect of *dld-1* gene suppression against Aβ-toxicity.

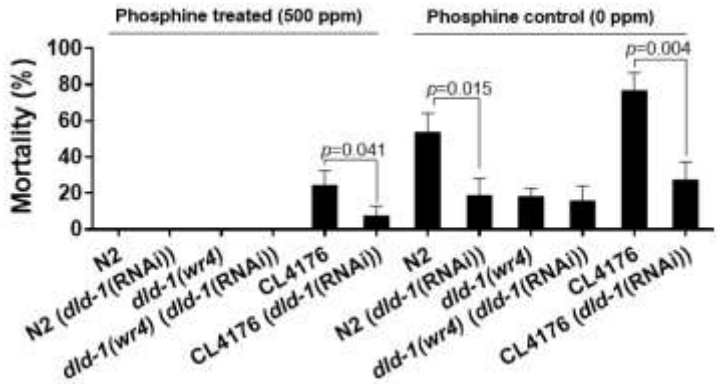

Figure 4.7: Suppression of the *dld-1* gene protects against the toxicity of phosphine independent of Aβ presence. *dld-1* gene mutation, or suppression by RNAi, reduced mortality

caused by 500 ppm phosphine in both wild type (N2) and Aβ-expressing worms of strain CL4176. Three independent trials were run (n=50-80 for each trial). Bars = mean ± SD.

The *dld-1*(*wr4*) mutation that is used in the current study confers resistance against phosphine toxicity [457] a phenotype that can also be achieved by *dld-1* gene suppression[409]. Phosphine is a fumigant that induces ROS production and lipid peroxidation but causes decreased respiration rates, as well as a reduction in mitochondrial membrane potential and ATP levels [458]. Thus phosphine, like Aβ, impairs mitochondrial function causing phenotypes that are countered by *dld-1* inhibition. Due to these similarities, we investigated interactions between *dld-1*, phosphine and Aβ.

We found that exposing Aβ expressing transgenic worms to 500 ppm phosphine (the LC_{50} of wildtype *C. elegans*) for 24 hours following by 48 hours of recovery at room temperature increased the mortality of the wildtype N2 strain to the same degree as the Aβ expressing strain CL4176 (Figure 7). Thus, mortality of N2 increased from 0% to 48.5±10.7% in response to 500ppm phosphine and mortality of CL4176 increased from 22.9±7.8% to 75.6±9.9%. The resistance phenotype of the *dld-1*(*wr4*) mutant was unaffected by RNAi-mediated suppression of the *dld-1* gene, indicating that the mutation and gene suppression confer resistance to phosphine by the same mechanism. The phosphine toxicity and Aβ toxicity is simply additive, regardless of whether or not the *dld-1* gene is suppressed. This indicates that while both phenotypes are affected by the DLD-1 enzyme, they are mediated independently without any interaction. Similar findings were observed when we treated the worms at a higher phosphine concentration of 2000ppm (Supplementary Figure S4).

Discussion

Imaging of the AD brain, consistently reveals a decrease in glucose metabolism in areas most strongly affected by disease pathology. While the effect of reduced glucose metabolism on AD progression has not been determined, the deposition of Aβ and reduction in glucose metabolism both begin decades before any clinical manifestation of the disease, indicating a possible causal relationship between the two [459].

To assess the role of energy metabolism in AD-associated Aβ proteotoxicity in worms, we used RNAi to suppress the activity of the *dld-1* gene, which is known to suppress aerobic respiration [460, 461]. The DLD enzyme is also a subunit of two mitochondrial enzyme complexes, pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (KGDH). These complexes contribute to the oxidative respiration of glucose and are implicated in AD as well [36, 212]. Our

results show that suppression of the *dld-1* gene significantly alleviates the symptoms associated with Aβ expression in either muscles or neurons of *C. elegans*.

We find that suppression of the *dld-1* gene does not affect either the *Aβ* transgene mRNA levels or the levels of Aβ peptide. Suppression of *dld-1* does, however, significantly inhibit the oligomerization of Aβ. Accumulation of Aβ oligomers is thought to be a major culprit in AD progression [462, 463], whereas monomers actually help to maintain glucose homeostasis and are not toxic [13, 464]. Our findings suggest that *dld-1* suppression reduces Aβ oligomerization thus resulting in reduced paralysis, better movement rates, and improved behavioral phenotypes as observed previously [57, 407, 465, 466].

Recent studies have observed that diet rich in carbohydrates accelerates neurodegeneration by increasing Aβ oligomers [467, 468]. Suppression of the *dld-1* gene causes a decrease in glucose catabolism, which would appear to have the opposite effect of a carbohydrate-rich diet. In fact, dietary restriction has been shown to delay Aβ-mediated proteotoxicity and extend lifespan in *C. elegans* by activating the DAF-16/ FOXO, AAk-2/AMPK and SIR-2.1/sirtuin pathways [274, 402, 469-471]. Both mutation and RNAi-mediated suppression of the *dld-1* gene result in phosphine resistance and an extended lifespan (Kim and Sun, 2007; Cheng et al., 2003; Schlipalius et al., 2012), as well as inhibition of Aβ oligomerization and protection against Aβ-mediated toxicity as we have shown here.

Based on our understanding of the relationship between the *dld-1* gene and phosphine toxicity/resistance, we carried out several additional assays designed to compare the mechanisms of action of *dld-1* and Aβ. When we exposed worms to FCCP, we found that a dose that had no effect on either wildtype or *dld-1*(*wr4*) mutant worms and only a modest effect on Aβ expressing worms was highly toxic when the *dld-1* gene was subjected to RNAi-mediated suppression. The basis of the interaction between FCCP and *dld-1* gene suppression is unknown. FCCP does, however, deplete the mitochondrial proton gradient that is utilized for ATP synthesis, whereas the DLD-1 enzyme generates NADH that delivers electrons to the electron transport chain that generates the proton gradient. It may be that simultaneous depletion of the proton gradient by FCCP as well as the source of electrons (NADH) by suppressing the *dld-1* gene, results in a cellular energetic catastrophe.

As protonophores, mitochondrial un-couplers also reduce the pH of the mitochondrial matrix. At low pH, the dehydrogenase activity of DLD is inhibited and the reverse activity (diaphorase) is induced [472, 473]. This would have the same effect on cellular energy metabolism

as described in the previous paragraph and indeed, both mechanisms may contribute to the synergistic increase in mortality that is observed.

Aβ proteotoxicity and oxidative stress are positively correlated [455, 474, 475] and DLD inhibition is known to reduce ROS generation [456, 476, 477]. The DLD enzyme and mitochondrial electron transport chain (ETC) are both major sources of ROS generation [297, 478], so ROS production could either be direct (less ROS emanating from DLD) or indirect (less NADH feeding electrons to the ETC). We found reduced levels of the mitochondrial superoxide detoxifying enzyme SOD-3 after *dld-1* gene suppression, suggesting that suppression of *dld-1* does indeed decrease the burden of ROS in *C. elegans*.

We also exposed worms to the fumigant phosphine, a mitochondrial poison that causes oxidative stress and inhibits respiration, likely by targeting the DLD enzyme [409, 458]. When combined, Aβ expression and exposure to phosphine cause an additive increase in mortality. Suppression of the *dld-1* gene protects against both Aβ and phosphine individually and provides the same degree of protection against the two stressors in combination. One interpretation of these results is that the two stressors act through the same mechanism(s), with each stressor simply increasing the magnitude of the insult.

The similarities that we observe between the toxicity of Aβ and phosphine are worth noting. Both cause suppression of energy metabolism and yet are protected by suppression of the *dld-1* gene, a manipulation that likewise suppresses energy metabolism. The toxicity of both Aβ and phosphine is synergistically exacerbated by co-exposure to the mitochondrial uncoupler, FCCP. However, co-exposure to Aβ and phosphine does not result in an additive rather than synergistically increased toxicity, an outcome that is consistent with Aβ and phosphine acting by the same mechanism.

Our findings establish a neuroprotective effect resulting from suppression of the *dld-1* gene that encodes a core enzyme of mitochondrial energy metabolism in *C. elegans* strains that express human Aβ. We find no evidence that metabolic suppression through DLD is a risk factor for Aβ proteotoxicity. Our results do not distinguish between two possibilities; that neuroprotection is a direct effect of metabolic suppression or that it is an indirect effect resulting from decreased ROS generation. Regardless of the mechanism, our results are consistent with the hypothesis that a decrease in mitochondrial energy metabolism protects against Aβ pathogenicity, which in humans could delay clinical dementia resulting from AD.

Supplementary information
Figure S4.1

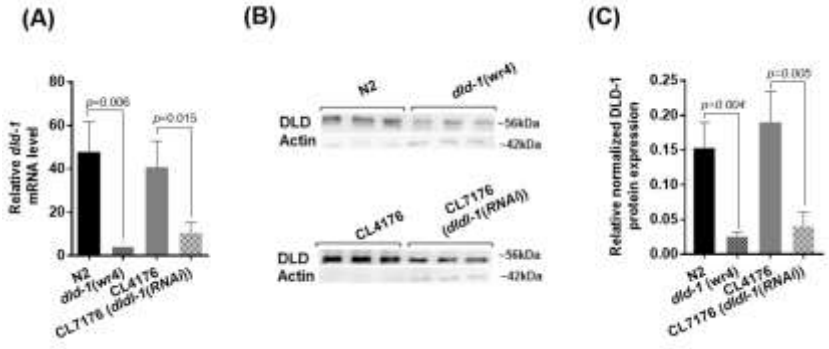

Figure S4.1: *dld-1* mutation, or suppression by RNAi causes a decrease in *dld-1* transcript and protein levels. Synchronized L1 stage worms of the Aβ expressing strain CL4176 were fed *E. coli* that expressed *dld-1* dsRNA for 36 hours at 16ºC. The temperature was raised to 25ºC for 36 hours to enhance Aβ expression. Temperature was also increased to 25°C in control worms (N2 and *dld-1*(*wr4*)) that do not express Aβ peptide. (A) Results of real-time quantitative PCR from three independent trials showing a significant decrease in *dld-1* mRNA levels in *dld-1* mutated and suppressed worms. (B) Western blot of protein extracted from cell lysate. Anti-lipoamide dehydrogenase antibody (ab133551) was used to detect DLD-1 protein, whereas anti-β-actin antibody was used as reference control. (C) Quantification of the DLD-1 bands from western blots using GelQuantNET software. Graphs and western blots represent the results from three independent experiments. Errors bars = mean±SD.

Real-time quantitative PCR and western blotting were used to confirm the efficacy of RNAi-mediated *dld-1* gene suppression. We assessed the *dld-1* mRNA and protein levels in the Aβ expressing strain CL4176 before and after *dld-1* suppression, compared to the wild type strain N2 and the *dld-1* mutant *dld-1*(*wr4*). When the *dld-1* gene was suppressed by RNAi, both transcript and protein decreased to the levels in *dld-1*(*wr4*) mutant (Figure S1A-S1C).

Figure S4.2

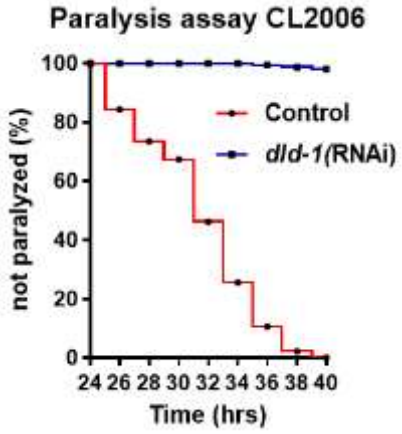

Figure S4.2: Effect of *dld-1* suppression on paralysis of the constitutively expressing Aβ strain CL2006. Synchronized worms were treated with *dld-1* RNAi for 36 hrs at 16ºC. The

temperature was shifted to 25ºC for the next 24 hrs and scored for paralysis every 2 hrs. Paralysis curves were assessed using Log-rank survival test.

Suppression of *dld-1* was found to delay the Aβ-associated paralysis in constitutively Aβ expressing worms just as occurred with the temperature inducible worms (Figure 1).

Figure S3

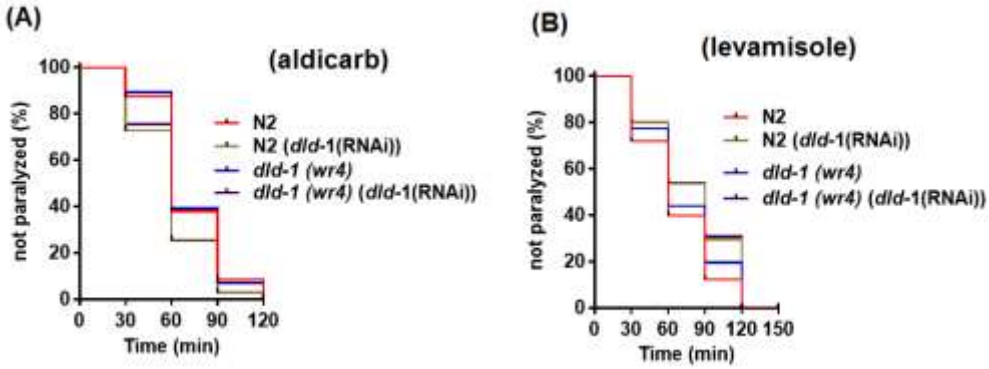

Figure S4.3: *dld-1* suppression does not change acetylcholine neurotransmission in wild type and *dld-1* mutated worms. *dld-1* was suppressed in Wild type N2 and the *dld-1* mutated worms using RNAi. After 72 hours of incubation at 20ºC, worms were shifted to NGM plates containing either; (A) 1mM aldicarb or (B) 0.2mM levamisole. A log-rank test was applied to made comparison among treatments.

The paralysis assay shows that *dld-1* gene suppression did not significantly affect acetylcholine neurotransmission in either wild type or *dld-1* mutant *C. elegans*. Thus, the improvement in acetylcholine neurotransmission observed in figure 2 was specific to the Aβ expressing strain, CL2006.

Figure S4

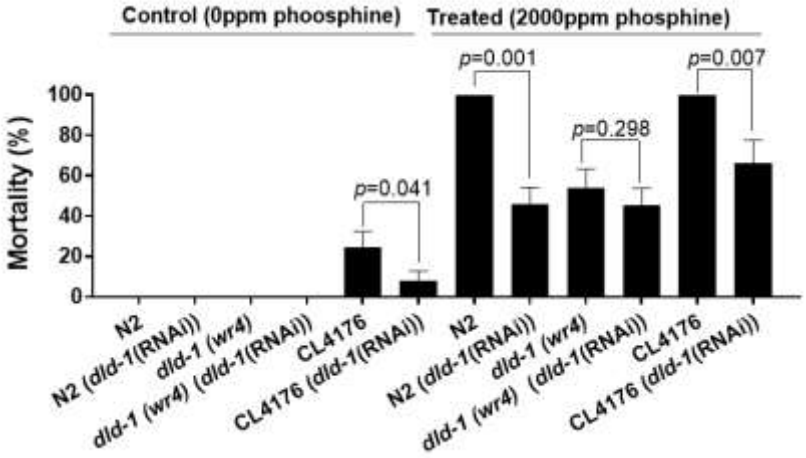

Figure S4.4: Suppression of *dld-1* gene protects against the toxicity of phosphine treatment at 2000ppm. Synchronized worms were either fed *E. coli* containing empty vector or expressing *dld-1* dsRNA. L4 stage worms were subjected to 2000ppm phosphine for 24 hours at 20ºC.

After a 48 hrs recovery time, worms were counted either dead or alive. Data are the result of three independent trials. Bars = mean± SD.

Wild type N2 and *dld-1(wr4)* exhibited no mortality in the absence of phosphine. The Aβ expressing strain CL4176 exhibited mild paralysis that was reduced by *dld-1* suppression (22.9±5.9% vs 7.1±5.2%, p=0.041). Phosphine treatment at 2000ppm (LC_{50} of the *dld-1*(*wr4*) mutant) killed 100% N2 and CL4176 worms, but only 53.7±9.1% of the *dld-1* mutant worms. *dld-1* suppression decreased the mortality rate in N2 (54.5±8.2, p=0.001) and CL4176 (67.4±11.5, p=0.007), but there was no significant effect observed on *dld-1*(*wr4*) mortality (45.3±8.23, p=0.298).

CHAPTER # V: 5-Methoxyindole-2-carboxylic acid (MICA) suppresses Aβ-mediated pathology in a *C. elegans*

Abstract

Alzheimer's disease (AD) is an age-related disease characterized by loss of memory and disrupted thinking that is associated with altered energy metabolism. Variants of an important metabolic gene, dihydrolipoamide dehydrogenase (*dld)* have been genetically linked to late-onset AD. Moreover, reduced activity of DLD-containing enzymes indicates an important role of energy metabolism in AD progression. To understand how energy metabolism influences AD progression, we exposed *C. elegans* expressing human Aβ peptide to the chemical inhibitor of DLD, 2-methoxyindole-5-carboxylic acid (MICA). We found that MICA alleviated the Aβ-induced paralysis and improved ACh neurotransmission in *C. elegans* that express Aβ in muscle cells. MICA also reduced both hypersensitivity to serotonin and perturbation of chemotaxis associated with neuronal expression of human Aβ. Furthermore, low doses of MICA improved fecundity. Protection against AD pathogenesis by MICA in the worm model was associated with a decrease in Aβ oligomerization and could be reversed by the calcium ionophore, A23187.

Introduction

We explored the effects of *dld-1* suppression by RNAi against Aβ- mediated toxicity in *C. elegans* (Chapter # IV of the current thesis). In this study we used a chemical inhibitor of DLD known as 5-methoxyindole-2-carboxylic acid (MICA) [479] to determine whether it also could alleviate Aβ toxicity in *C. elegans* that express human Aβ.

Evidence indicates that impaired energy metabolism is an important cause of AD. Furthermore, having the metabolic disease diabetes increases the risk of AD [50, 480, 481]. As both AD and diabetes share many pathological features [1, 51], it is possible that drugs used to lower hyperglycemia might be effective in AD treatment. MICA is a hypoglycemic agent that has been suggested as possible drug to treat T2DM [482-486]. MICA modifies cellular glucose levels by altering glucose metabolism but not insulin sensitivity as MICA was unable to induce glucose or galactose uptake by liver, pancreas or diaphragm peripheral tissues of the mouse [482, 487, 488].

Although the exact mechanism of action of MICA is not fully understood, MICA inhibits the oxidation of keto acids whose catabolism is dependent on DLD-1 containing enzyme complexes. Meanwhile, MICA also inhibits the carboxylation of pyruvate thus reducing glucose production from non-carbohydrate carbon sources [486, 488]. MICA was found to suppress the

substrate-driven decrease of α-lipoate, a cofactor in the keto acid catabolizing, DLD-1 containing enzyme complexes. This was reflected in reduced DLD enzymatic activity in mitochondria [486, 489]. The inhibitory effects of MICA could be reversed *in vitro* by the addition of lipoic acid in the media [486] or by increasing intracellular calcium levels using the calcium ionophore A23187 (CaI) [490, 491]. Although the exact mechanism in not known CaI was found to induce the levels of lactate dehydrogenase (LDH) [492, 493] that ultimately increase pyruvate production from lactic acid thus induce downstream energy metabolism.

Little is known about other effects of MICA on the cellular system. Other indole derivatives do not directly affect DLD activity, but many influence energy metabolism indirectly. For example, indole derivatives have been identified that reduce aerobic glycolysis and formation of pyruvate from lactate thus slowing down energy metabolism [494, 495].

Currently, there is no evidence about the role of MICA on neurodegeneration. However, it is possible that MICA may influence neurodegeneration by modifying energy metabolism. Our results showed that MICA protects against Aβ-mediated toxicity in *C. elegans* that express human Aβ. The protective effect of MICA is associated with a decrease in Aβ oligomerization. In our study, the protective effects of MICA can be reversed using CaI.

Results

How energy metabolism affects Alzheimer's disease is still unclear. However, a positive correlation exists between decline in glucose metabolism and age as well as the progression of late-onset AD. Two mutually exclusive hypotheses have been proposed, that reduced energy metabolism exacerbates disease progression or that it is a protective response that slows the progression of the disease. Our previous work indicated that suppression of energy metabolism by RNAi-mediated suppression of the *dld-1* gene results in significant protection against Aβ proteotoxicity Based on this finding, we reasoned that the DLD-1 enzyme inhibitor 2-methoxyindole-5-carboxylic acid (MICA) might act as a small molecule therapeutic agent in the treatment of AD. Here we show that MICA reduce Aβ-mediated, age-dependent paralysis, impaired ACh neurotransmission, and impaired behaviour in worm models of AD.

MICA up to 10mM does not alter dld-1 gene or protein expression

MICA is a known inhibitor of the DLD enzyme [496], but its effect on *dld-1* transcript and protein levels is not known. We find that *dld-1* RNAi was able to suppress the *dld-1* transcript levels in the

CL4176 strain (p=0.015), whereas exposure to MICA caused no significant change (p=0.172) (Figure 5.1A).

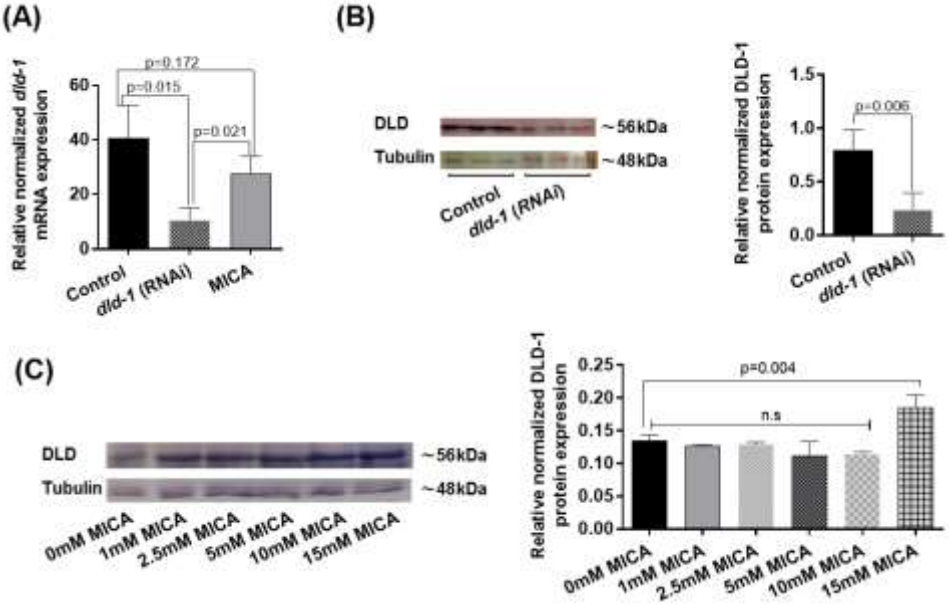

Figure 5.1: MICA does not affect *dld-1* transcript levels. Synchronized L1 staged muscular ~ temperature inducible Aβ expressing worms of CL4176 strain were treated with different concentrations of MICA or fed with *dld-1* ds-RNAi expressing *E. coli* for 36 hrs at 16ºC, followed by 36 hrs at 25ºC to induce transgene expression before RNA or protein extraction. (A) Quantification of relative *dld-1* mRNA levels using RT-PCR from three independent experiments. (B) Immunoblot assay of total soluble protein from cell lysate. DLD-1 protein expression was detected using the anti-DLD antibody 6E10 while anti-tubulin was used as reference control to monitor equal loading of protein. (C) Immunoblot assay of total protein extracted from worms treated with different concentrations of MICA. Anti-DLD antibody was used to detect DLD-1, while anti- tubulin antibody served as reference control. Graphs show results from three independent replicates. Bars = Mean ±SD.

RNAi of the *dld-1* gene significantly suppressed the DLD-1 protein abundance in the CL4176 strain (Figure 5.1B, p=0.006). Our results showed that concentrations of MICA from 1-10mM have no significant effect on DLD-1 protein expression (p>0.05), whereas 15mM MICA causes a small increase (p=0.004) (Figure 5.1C). These results will aid in the interpretation of MICA effects in the remainder of the manuscript.

MICA alleviates Aβ- induced pathology in transgenic C. elegans

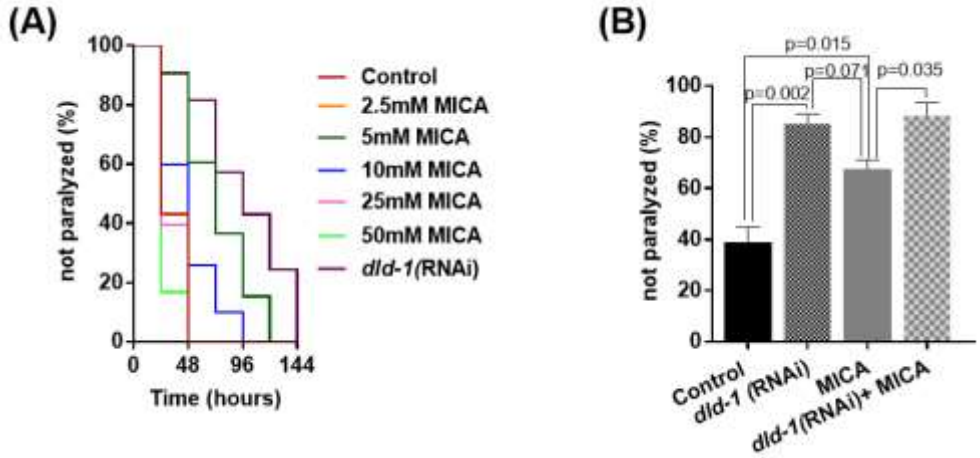

Figure 5.2: MICA reduces paralysis due to Aβ muscular expression in worms. Temperature inducible Aβ expressing worms were treated with MICA at concentrations ranging from 2.5 to 50mM on normal NGM plates soon after hatching. For *dld-1 gene* suppression, plates were seeded with HT115 (empty vector) or with *dld-1* ds-RNAi expressing *E. coli* (A) Time in hours after inducing Aβ expression by temperature up-shift to 25ºC in strain CL4176. B) Co-effect of 5mM MICA and *dld-1* suppression by RNAi after 24 hrs of temperature induction. Bars= mean ± SD. Averaged percentage values of active worms between treatments from three independent trials containing 60-80 worms per plate were compared using log-rank survival test.

In our study, MICA was not able to reduce *dld-1* gene and protein expression. It has, however, been known to inhibit DLD protein activity and reduce downstream energy metabolism [32, 496]. The suppression of *dld-1* by RNAi in *C. elegans* is associated with stress resistance [46, 409]. We also demonstrated in chapter 4 that *dld-1* gene suppression inhibits the oligomerization and toxicity of Aβ. We hypothesized that chemical inhibitors of the DLD-1 enzyme might also protect against Aβ-toxicity in Aβ expressing worms.

Aβ-mediated toxicity in worms is associated with age-dependent paralysis [55]. To determine whether MICA can delay Aβ-mediated paralysis, worms of strain CL4176 that contain a temperature inducible Aβ transgene were exposed to MICA continuously, before, during and after induction of Aβ expression. The time to 100% paralysis of the 0mM MICA control was 48 hours. Both 5mM and 10mM MICA significantly delayed the time to paralysis, though the 5mM treatment had the greatest effect (120 hours, $p<0.0001$). Interestingly, at low (2.5mM) and at high (25mM) concentrations, MICA had no effect on paralysis ($p>0.05$), whereas 50mM MICA accelerated paralysis as most worms become paralyzed within 24 hours ($p=0.01$) (Figure 5.2A).

As DLD-1 inhibition by MICA and suppression of the *dld-1* gene by RNAi can both alleviate Aβ-mediated paralysis, we tested the two treatments in combination to see how they interacted. To accomplish this, we compared the effect after 24 hours of 5mM MICA and *dld-1*

gene suppression individually as well as in combination. Suppression of the *dld-1* gene doubled the number of active worms at 24 hours. MICA also increased the proportion of active worms to a level that was statistically indistinguishable from *dld-1* gene suppression (67.5 ± 3.7 vs 85.5 ± 3.5, p=0.071).

Aβ expression in worms impairs cholinergic neurotransmission and associated functions like muscle contraction and movement [497]. In worms where the presence of Aβ blocks the release of ACh, acute paralysis caused by hyper excitation of the cholinergic system is delayed. Thus, acute paralysis caused by two cholinergic agonists, aldicarb, an ACh esterase inhibitor (pre-synaptic effect) and levamisole, a receptor agonist (post-synaptic effect) is delayed in strains that express Aβ [407, 465]. To check whether MICA restores normal ACh neurotransmission (i.e. susceptibility to acute paralysis by cholinergic agonists), the CL2006 strain that constitutively expresses Aβ in muscle cells were exposed to aldicarb or levamisole.

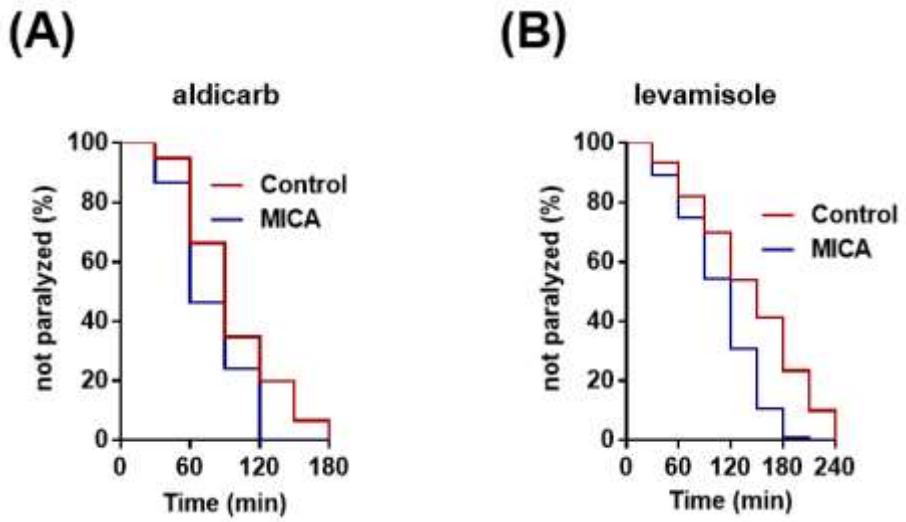

Figure 5.3: MICA reinstates ACh neurotransmission in constitutively muscular Aβ expressing worms. Strain (CL2006) was exposed to 5mM MICA for 72 hrs and then shifted to 5mM MICA-NGM plates containing 1mM aldicarb or 0.2mM levamisole. Control worms were treated without MICA. (A) Time dependent paralysis of MICA treated or untreated worms in presence of aldicarb. (B) Time dependent paralysis of MICA treated or untreated worms in presence of levamisole. For each set of experiment, every group contains at least 30-50 worms per plate. Three independent trials were run for each assay. Paralysis curves were compared using log-rank test. Bars = Mean ± SD.

In our study, MICA restored sensitivity to aldicarb, resulting in paralysis within 120 min compared to worms that were not exposed to MICA (180 min, p<0.0001) (Figure 5.3A). In contrast, paralysis due to the postsynaptic receptor agonist, levamisole, was only decrease from 240 min to 210 min (p=0.0001) after MICA treatment (Figure 5.3B). As MICA did not change the paralysis profile of either wild type or *dld-1* mutated worms (Figure S5.1), the increase in ACh neurotransmission is most likely the result of a decrease in cholinergic inhibition by Aβ.

We next determined whether MICA was also effective at reversing the negative effects of Aβ expression in the nervous system using strain CL2355 and its matched control CL2122. In AD, Aβ is primarily expressed in neurons. Assays for cellular dysfunction due to neuronal expression of Aβ in *C. elegans* include impaired chemotaxis, serotonin hypersensitivity, and egg laying and hatching [57, 498].

Neuronal expression of Aβ significantly reduced chemotaxis toward the attractant benzaldehyde compared to no-Aβ control worms (Chemotaxis index, 0.062 ± 0.014 vs 0.219 ± 0.06, p=0.004). MICA treatment significantly restored chemotaxis of strain CL2355, despite neuronal expression of Aβ (0.062 ± 0.014 vs 0.138 ± 0.026, p= 0.018), which was close to the control value (, p=0.121) (Figure 5.4A). MICA did not affect chemotaxis in the no-Aβ control worms CL2122 (0.185 ± 0.035, p=0.70).

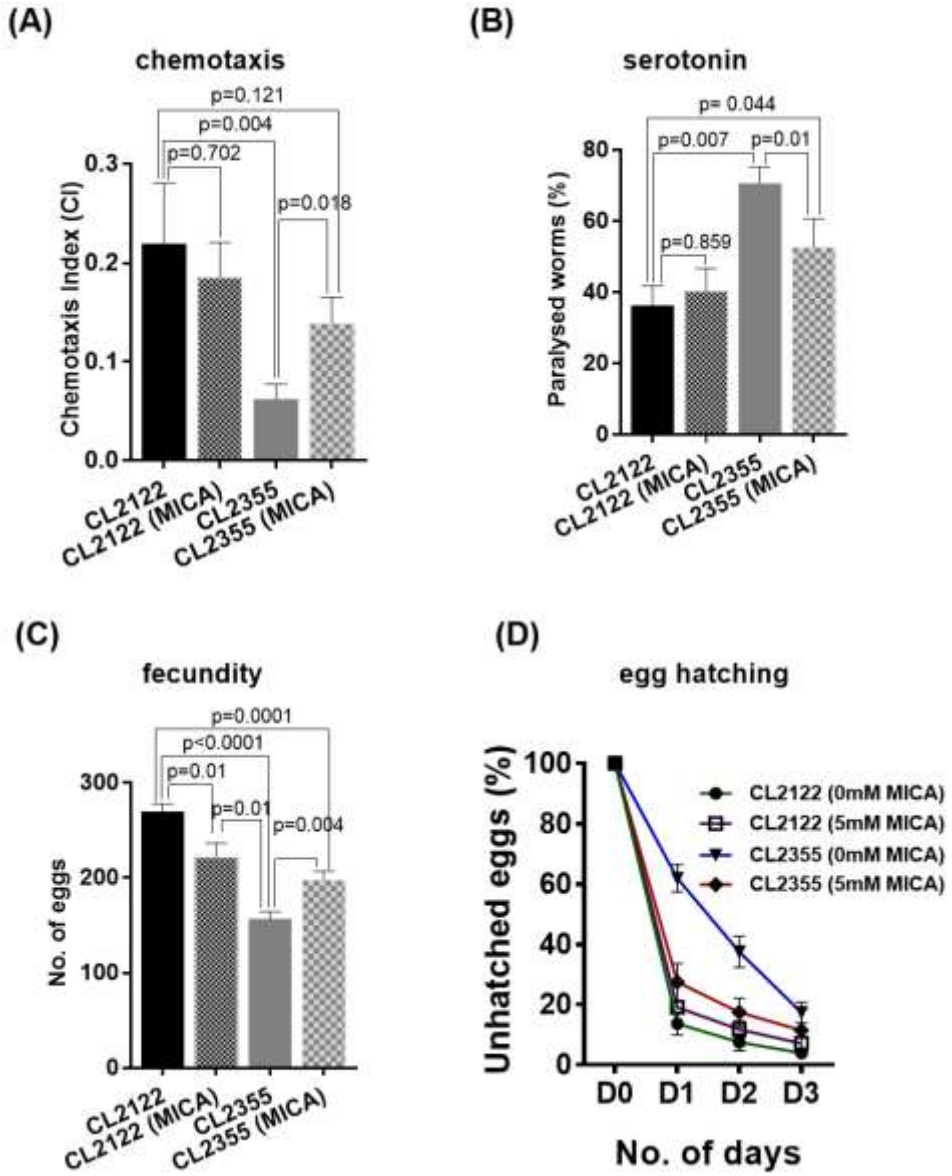

Figure 5.4: Effect of MICA on impaired chemotaxis, serotonin sensitivity and fecundity in neuronal-Aβ expressing worms. Neuronal Aβ expressing worms CL2355 and their matched control CL2122 were synchronized and placed at normal or 5mM MICA containing NGM plates for 36 hrs at 16ºC and subsequently shifted to 25ºC for next 36 hrs to induce transgene expression. (A) Analysis of impaired chemotaxis by MICA treatment. (B) Assessment of 5-HT serotonin hypersensitivity in Aβ- neuronal expressing worms after MICA exposure. (C) Fecundity measurement in neuronal-Aβ expressing worms treated with or without MICA. (D) Evaluation of egg hatching time in neuronal-Aβ expressing worms after MICA treatment. For each set of experiment, every group contains at least 30-50 worms per plate except for fecundity and egg hatching assays where 10 worms were used in each trial. Three independent trials were run for each assay. Bars = Mean ± SD.

C. elegans exposed to exogenous serotonin become paralyzed whereas neuronal expression of Aβ makes them hypersensitive to serotonin when compared to matched control [57]. Exposure to serotonin increases paralysis due to Aβ expression compared to no-Aβ control worms of strain CL2122 (70.6±4.5% vs 36.3 ± 5.5%, p=0.007). MICA did not change the paralysis level of the CL2122 worms (36.3 ± 5.5% vs 40.3±6.3%, p=0.859) but was able to reduce paralysis in strain CL2355 in which Aβ was expressed (70.6±4.5% vs 52.6±8.1, p=0.01). The fraction of CL2355, MICA-treated worms that were paralyzed, however, did not return to the CL2122 MICA-treated control level (52.6±8.1 vs 40.3±6.3%, p=0.044) (Figure 5.4B).

The presence of Aβ is also known to decrease fecundity and increase egg hatching time in *C. elegans* [498], so we tested whether MICA could reverse these effects. The fecundity of the Aβ strain CL2355 was indeed lower than its matched control (157±7.5 vs 270±7.9, p< 0.0001) (Figure 5.4C). 5mM MICA significantly improved fecundity in Cl2355 worms (157±7.5 vs 198±8.8, p= 0.004). While this increase did not reach the level of the untreated control (198±8.8 vs 270±7.9, p=0.0001). Despite an increase in egg laying in strain CL2355 that expressed Aβ, MICA surprisingly reduced fecundity in CL2122 control worms (270±7.9 vs 222±14.5, p=0.01). A higher dose of MICA (10 mM) caused a decrease in fecundity for both strains (Figure S5.2).

Aβ expression also impedes egg hatching in worms. Only 13.7% of matched control (CL2122) eggs remained unhatched after 24 hrs when compared to Aβ transgenic strain CL2355 (61.8%, p<0.0001) (Figure 5.4D). Hatching was greatly improved in the CL2355 strain after exposure to 5mM MICA, with only 27.5% of CL2355 worms remaining unhatched after 24 hours (p=0.001). Almost 92% of eggs from control worms hatched by day 2 however, only 62.4% of eggs from the Aβ transgenic strain hatched (p=0.0009). In contrast, Aβ transgenic worms treated with 5mM MICA showed an increase in egg hatching to 82.2% (p=0.007) on day 2. In Aβ transgenic worms, egg hatching was impeded such that 17.5%, were still unhatched on day 3.

Protective effects of* DLD-1 *inhibition can be reversed by calcium ionophore (CaI)

Although we found that MICA can protect against Aβ, it is toxic and has a narrow effective dose range [483, 488]. If MICA is to be used effectively against AD, it is imperative that we understand the basis of its toxicity. One observation that deserves investigation is that MICA can influence calcium signaling. For example, inhibition of DLD by MICA causes a decrease in intracellular calcium that can be reversed by the calcium ionophore A23187 (CaI) [499]. This finding raises the possibility that CaI might also reverse the effects of *dld-1* inhibition on strains that express Aβ. To test this hypothesis, we assessed the effect of CaI on Aβ-mediated paralysis alone or in the presence of either 5mM MICA or *dld-1* gene suppression by RNAi.

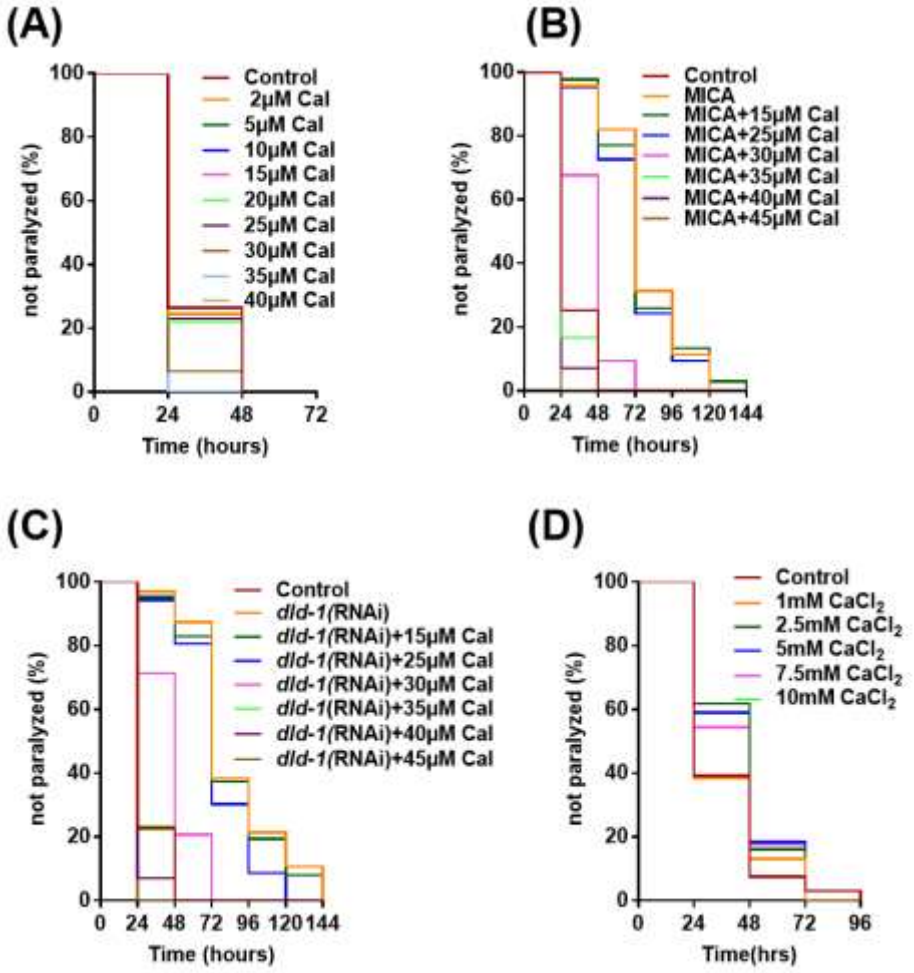

Figure 5.5: Calcium ionophore (CaI) reverse the MICA effects. After synchronization, temperature inducible Aβ- muscular expressing worms CL4176 were treated with different concentrations of CaI in the absence or presence of 5mM MICA or *dld-1* RNAi, or with different concentrations of calcium chloride ($CaCl_2$), and assessed for paralysis. (A) Effect of CaI on Aβ-mediated paralysis in CL4176 worms. (B) Feeding of worms with distinct concentrations of CaI in the presence of 5mM MICA. (C) Exposure of worms to different concentrations of CaI with *dld-1* RNAi suppression. (Worms fed on *E. coli* containing empty vector (HT115) were used as control (D) Aβ expressing worms CL4176 were cultured on NGM agar, supplemented with various concentrations of $CaCl_2$. The temperature for this

assay was 24ºC. Results are derived from the three independent replicates and given as the average. Curve comparison was completed using long-rank test. 60-80 worms were used for each trial.

Doses of CaI up to 30 µM have no effect on Aβ-mediated paralysis, whereas higher doses increased paralysis (Figure 5.5A). For Aβ-expressing worms treated with a maximally protective dose of 5 mM MICA, a CaI dose of 30µM partially reversed the protective effect. At 35µM CaI paralysis was equivalent to that observed in Aβ expressing control worms without MICA treatment (Figure 5.5B). 35µM CaI also completely reversed the protection against Aβ expression provided by suppression of the *dld-1* gene through RNAi (Figure 5.5C).

Our results showed that increase in intracellular Ca^{2+} levels using CaI significantly overturn the DLD-1 suppression. However, we observed no change in paralysis profile of Aβ expressing worms after addition of $CaCl_2$ up to 10mM in the media (Figure 5.5D).This result shows that increase in extracellular Ca^{2+} might have no effect on intracellular Ca^{2+} levels.

MICA and CaI modulate Aβ protein oligomerization without affecting Aβ peptide levels

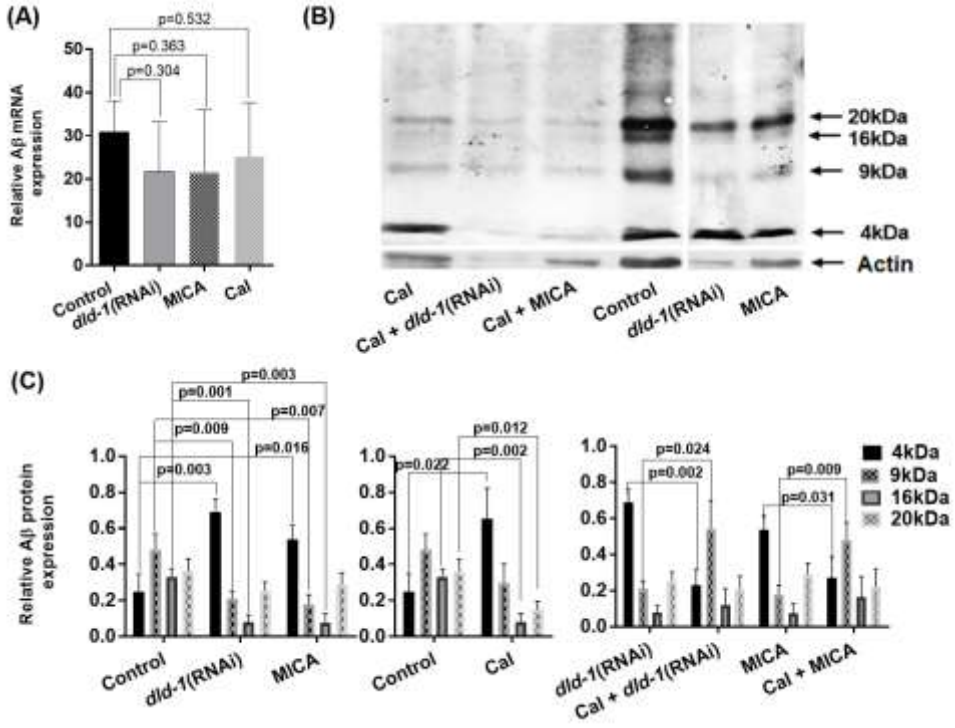

Figure 5.6: Effect of MICA and CaI on Aβ transgene and protein expression. Temperature inducible Aβ- muscular expressing worms were synchronized and placed at NGM plates with and without *dld-1* RNAi or 5mM MICA or 35 µM CaI or the combination of *dld-1* RNAi plus

CaI or MICA plus CaI for 36hrs at 16ºC. The temperature was induced at 23ºC for 36 hrs before RNA or protein extraction. (A) Results of quantitative PCR showing effect of *dld-1* RNAi, 5mM MICA and CaI treatments on Aβ mRNA levels. (B) Western blotting of Aβ deposits in transgenic worms CL4176. Anti-Aβ antibody 6E10 and/ or anti-actin (reference) were used to detect the proteins. C) Quantification of Aβ levels relative to reference control using GelQuantNET software. To ease calculations and understanding, quantitative graph was divided into three sections. Graphs represent results from three independent trials. Bars = Mean±SD.

In our study, MICA successfully reduced the Aβ toxicity in worms that express Aβ in either muscle cells or neurons of *C. elegans*. MICA is known to inhibit DLD-1 enzyme activity and CaI reverse this suppression, but we wished to confirm that there were no confounding effects on Aβ gene expression that would lead us to misinterpret how MICA and CaI might influence the *C. elegans* model of Aβ. Quantitative PCR revealed no change in Aβ mRNA levels after RNAi-mediated suppression of the *dld-1* gene, treatment with MICA, or exposure to CaI (Figure 5.6A).

We also monitored to determine whether there was any effect of these interventions on oligomerization of Aβ as increased oligomerization induced by CaI is one way that it could overcome the protective effect of the other treatments [13]. We used the Aβ specific antibody (6E10) on western blots to detect Aβ monomers and oligomers (Figure 5.6B). Figure 5.6C represents quantification of respective band intensities normalized to the amount of β-actin in each protein sample from three independent experiments.

Our results showed that MICA significantly increases the abundance of Aβ monomers (~4kDa: 0.24±0.09 vs 0.53±0.07, p=0.016), while simultaneously decreasing the abundance of Aβ oligomers (~9kDa: 0.48±0.08 vs 0.17±0.0.05, p=0.002 and ~16kDa: 0.33±0.04 vs 0.07±0.0.05, p=0.002) in the transgenic Aβ expressing strain CL4176. Similar results were observed in CL4176 worms in which the *dld-1* gene had been suppressed by RNAi. There was an increase in ~4kDa monomers (0.24±0.09 vs 0.68±0.07, p=0.003) and reduction in ~9kDa (0.48±0.08 vs 0.21±0.04, p=0.002) and ~16kDa (0.33±0.04 vs 0.07±0.03, p=0.002) oligomers. It was interesting to see that on its own, 35μM CaI does not increase Aβ oligomerization, but rather decreases it. Aβ ~16kDa oligomers decreased from 0.30±0.04 to 0.02±0.07, p=0.002 in response to CaI and ~20kDa oligomers decreased from 0.33±0.04 to 0.07±0.04, p=0.012. At the same time, the abundance of ~4kDa monomer increased from 0.24±0.09 to 0.65±16, p=0.02. However, in conjunction with either *dld-1* RNAi or MICA, CaI induced oligomerization of Aβ at 9kDa, and decreases 4kDa monomers (Figure 5.6B and 5.6C).

Discussion

In this study, we had the potential therapeutic role of MICA on Aβ-induced toxicity in *C. elegans*. MICA has been known as a potent DLD inhibitor [491, 496] while the activities of key mitochondrial enzymes containing DLD like PDH and KGDH have been diminished in AD [36-38, 500]. As a subunit of enzyme complexes KGDH and PDH, our observations on the role of DLD in the *C. elegans* supported the growing awareness of a fundamental role of energy metabolism in the initiation and progression of AD. Mutation in the *dld-1* gene in *C. elegans* results in stress resistance and an extended lifespan [408, 409] that could be replicated by suppression of the *dld-1* gene by RNAi [46, 408, 409]. The obvious extension of these observations was to test MICA, a chemical inhibitor of DLD, to determine whether it had therapeutic properties on Aβ-mediated toxicity or not.

In our study, MICA did not affect the *dld-1* gene as well DLD-1 protein expression in *C. elegans*. Although MICA is known as a potent inhibitor of DLD enzymatic activity, however, blocking enzymatic activity does not mean protein expression will be reduced. The possible reason behind the increase in DLD-1 levels after MICA treatment could be a reaction of living system to balance the enzyme-substrate ratio. Our results showed that MICA can lessen symptoms triggered by Aβ expression in muscle. MICA delayed the time-dependent paralysis and improved ACh neurotransmission. We observed dose dependent inhibition of DLD activity by MICA as described earlier [491] and higher MICA concentrations resulted in induced paralysis. Exposure to MICA also restored the sensitivity of Aβ expressing worms towards ACh activators. The resistant to ACh inducers in Aβ transgenic control was proposed as the result of accumulation of Aβ oligomers in muscles cells that block ACh release into the synaptic cleft [57, 137]. While MICA did not affect the paralysis profile in no-Aβ control phenotype, we suggest that these effects are associated with modulation of Aβ expression.

Like muscular impairment, Aβ expression in *C. elegans* neuronal cells is associated with many behavioral defects like reduced chemotaxis, serotonin hypersensitivity, and impaired fecundity and egg hatching [57, 498]. The defects in these behaviors can be associated with impaired ACh signaling [497]. As MICA restored cholinergic neurotransmission in worms that express Aβ in muscle cells, we reasoned that MICA might also reduce Aβ-mediated toxicity in worms that express Aβ in neurons. In our previous study, we found that metformin, a well-known hypoglycemic agent, improved chemotaxis and restored normal serotonin-sensitivity in worms that express Aβ in neurons [2]. Similar findings were observed for MICA in this study suggesting that the two compounds have a similar mode of action. Accumulation of Aβ, reduced cholinergic

neurotransmission and impaired calcium signaling have been proposed as causes of serotonin-hypersensitivity in worms that express Aβ. ACh negatively regulates serotonin activity and the presence of Aβ not only blocks ACh but also block serotonin re-uptake [57]. Chemotaxis in *C. elegans* is controlled by serotonin signaling, making it a suitable indicator of proper serotonin signaling [501]. MICA has no affinity for serotonin receptors [502] and has no effect on serotonin sensitivity in no-Aβ controls. Thus, the ability of MICA to enhance chemotaxis and restoration of normal serotonin sensitivity in transgenic worms is most likely due to a decrease in Aβ pathology leading to improved ACh neurotransmission.

Despite impairing neuronal controlled behaviors, Aβ expression also reduce fecundity and increased egg hatching time in *C. elegans* [498]. Although MICA improved fecundity and reduced egg hatching time in Aβ expressing worms, it was not up to the matching controls. In previous studies, MICA was found to decrease fertility [499]; thus MICA should result in reduced fecundity and egg hatching in worms also. Here we postulated that induced egg laying in Aβ worms was due to modulation of Aβ protein while further addition of MICA reversed this process by blocking DLD-1 that resulted in decreased egg laying and a delay in egg hatching.

Our above-mentioned results showed that MICA reduced the Aβ-proteotoxicity in *C. elegans*. To understand the possible mechanisms involved, we assessed the role of calcium signaling on MICA-induced protection against Aβ-toxicity. We further evaluated the effect of MICA and calcium signaling on Aβ production and oligomerization. The beneficial effects of exposure to MICA might be mediated through calcium signaling. Indeed, the calcium ionophore (CaI) increased Aβ-mediated paralysis dose dependently. Addition of CaI also reversed the protective effects of *dld-1* gene suppression as well as DLD-1 enzyme inhibition by MICA [499, 503]. CaI is believed to interfere with MICA by increasing intracellular calcium levels [486, 499]. Impaired calcium homeostasis in aging brains and neurodegenerative disorders is well documented where increased levels of calcium accelerate Aβ toxicity [473, 504, 505].

We also tested the effect of an increase in extracellular calcium on Aβ-proteotoxicity, but increasing the calcium in the growth medium from 1mM to 10mM had no effect on the Aβ-mediated paralysis phenotype. This result indicates that the external calcium pool has minimal effect on intracellular calcium levels in the worm.

In our study, MICA did not influence the *A*β mRNA levels, but rather inhibited the formation of oligomers, resulting in a corresponding increase in the level of Aβ monomers. This change from Aβ oligomers to monomers has been shown in other studies to reduce Aβ toxicity [55-

57, 405, 413, 419, 465]. CaI induced Aβ oligomerization in worms in which oligomerization had been suppressed by RNAi of the *dld-1* gene. This provides a possible mechanism whereby CaI can reverse the protective effect of *dld-1* RNAi against the toxicity of Aβ. Surprisingly, CaI reduced oligomerization of Aβ in control worms that expressed Aβ, but in which the *dld-1* gene was not suppressed by RNAi. Matching results were also reported by Tauffenberger et al [506] where high glucose concentrations protect against Aβ-mediated paralysis but simultaneously induced the formation of toxic oligomers of Aβ. Overall, the protective effect of MICA seems to occur through inhibition of Aβ oligomerization, which can be modulated through calcium signaling.

Although MICA was suggested as a possible drug for diabetes 65 years ago, but due to significant side-effects, little effort has been made to test this compound for the treatment of diabetes or other metabolic disorders. We demonstrate that MICA provides significant protection against Aβ-mediated pathology in the *C. elegans* model of AD. This suggests that the wide array of commercially available MICA derivatives should be investigated as potential therapeutic compounds for the treatment of AD.

Supplementary Information
Figure S5.1

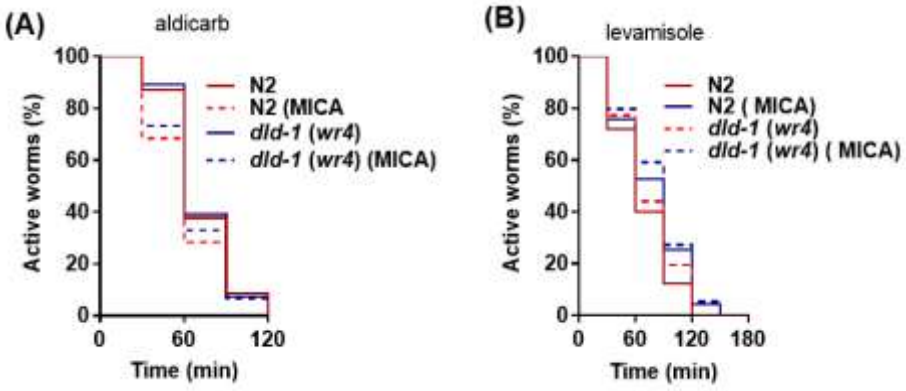

Figure S5.1: Effect of MICA on acetylcholine neurotransmission in wild type and *dld-1* mutated worms. Wild type and the *dld-1* mutant worms were treated with 5mM MICA. After 72 hours of incubation at 20°C, worms were shifted to NGM plates containing 1mM aldicarb or 0.2mM levamisole in the presence of 5mM MICA.
Our results showed that 5mM MICA did not affect acetylcholine neurotransmission in these strains.

Figure S5.2

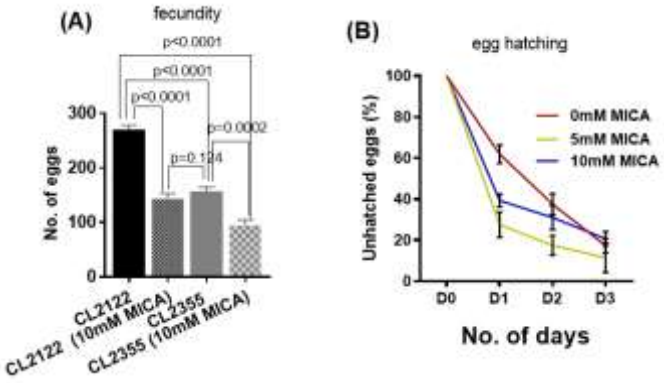

Figure S5.2: Effect of 10mM MICA on fecundity and egg hatching. (A) Fecundity measurement in Aβ neuronal expressing stain CL2355 and their matched control CL2122. (B) Time-course of egg hatching in CL2355 fed on 0mM, 5mM and 10mM MICA, respectively.
An increase in MICA significantly reduced fecundity in CL2122 (270±7.9 vs 144±8.1, $p<0.0001$) and CL2355 (198±8.8 vs 94.1±10.5, $p=0.0002$). Although mostly eggs were hatched earlier after MICA treatment, 10mM MICA reduced the egg hatching capability in CL2355.

CHAPTER # VI: High glucose levels facilitate Aβ oligomerization and tau phosphorylation in *C. elegans* model of Alzheimer's disease

Abstract

Formation of Aβ plaques from peptide oligomers and development of neurofibrillary tangles from hyperphosphorylated tau are hallmarks of Alzheimer's disease (AD). These markers of AD severity are further associated with impaired glucose metabolism. However, the exact role of glucose metabolism on disease progression has not been elucidated. In this study, the effects of glucose on Aβ and tau-mediated toxicity are investigated using a *C. elegans* model system. We find that addition of glucose or 2-deoxy-d-glucose (2DOG) to the growth medium delayed Aβ-associated paralysis, though it was unable to restore previously impaired acetylcholine neurotransmission in pre-extisting Aβ-mediated pathology. Glucose also inhibited egg laying and hatching in the Aβ-expressing worms. The harmful effects of glucose were associated with an increase in toxic Aβ oligomers. Increased phosphorylation of tau is associated with formation of neurofibrillary tangles (NFTs) and increased severity of AD, so we reasoned that high glucose levels might induce tau O-β-GlcNAcylation, thereby protecting against tau phosphorylation. Contrary to our expectation, glucose increased tau phosphorylation but not O-β-GlcNAcylation. Increasing O-β-GlcNAcylation, either with Thiamet-G (TMG) or by suppressing the O-GlcNAcase (*oga-1*) gene does interfere with and therefore reduce tau phosphorylation. Furthermore, reducing O-β-GlcNAcylation by suppressing O-GlcNAc transferase (*ogt-1*) gene causes an increase in tau phosphorylation. These results suggest that protective O-β-GlcNAcylation is not induced by glucose. Instead, as with vertebrates, we demonstrate that high levels of glucose exacerbate disease progression by promoting Aβ aggregation and tau hyperphosphorylation, resulting in disease symptoms of increased severity. The effects of glucose cannot be effectively managed by manipulating O-β-GlcNAcylation in the tau models of AD in *C. elegans*. Our observations suggest that glucose enrichment is unlikely to be an appropriate therapy to minimize AD progression.

Introduction

Our results (chapter IV and V of this study) showed that DLD-1 suppression could reduce the downstream glucose dependent energy metabolism. In this study, we directly tested the effect of glucose enrichment on Aβ and tau toxicity in *C. elegans* models expressing either human Aβ or tau.

Most AD therapies focus on decreasing the Aβ levels and tau hyperphosphorylation. Hyperphosphorylation of tau leads to self-aggregation into tangles of filaments that cause a loss of neuronal function and neuronal degeneration [507, 508]. Tau O-β-GlcNAcylation is reciprocally

antagonistic to phosphorylation and has been proposed as a possible therapy to reduce tau phosphorylation [507, 509, 510]. Glucose metabolism through the hexosamine biosynthesis pathway was proposed to induce O-β-GlcNAcylation, whereas a decrease in intracellular glucose levels were suggested to be associated with reduced O-β-GlcNAcylation and increased tau phosphorylation in AD brains [226, 229]. However, no study has directly tested the effect of elevated glucose levels on tau O-β-GlcNAcylation and phosphorylation.

An opposing model of the interaction between glucose and AD involves energy metabolism. In one iteration, this energy metabolism model suggests that impaired glucose metabolism causes AD. This is supported by the observation that reduced glucose metabolism and associated enzyme activities were observed in AD patients [41, 433-435, 437, 511]. In contrast, there is a decrease in Aβ-toxicity after caloric restriction or inhibition of glucose catabolism, which indicates that reduction in glucose-associated metabolism protects against neurodegeneration [31, 32, 45, 401, 402, 438, 512]. These contradictory observations mark glucose metabolism as an important target for studies designed to increase our understanding of the progression of AD.

A drug known as Thiamet G (TMG) that induces O-β-GlcNAcylation was also used in our study along with glucose to examine the effect of O-β-GlcNAcylation on Aβ toxicity and tau phosphorylation.

Results

Glucose is an essential source of energy generation in neurons whereas a decline in glucose metabolism was evident in patients with AD. Here in this study, we assessed any possible link between Aβ and tau-mediated toxicity and glucose enrichment using transgenic *C. elegans* strains that express the human peptides.

Glucose, glycerol, and 2DOG delay paralysis caused by Aβ expression in C. elegans muscle

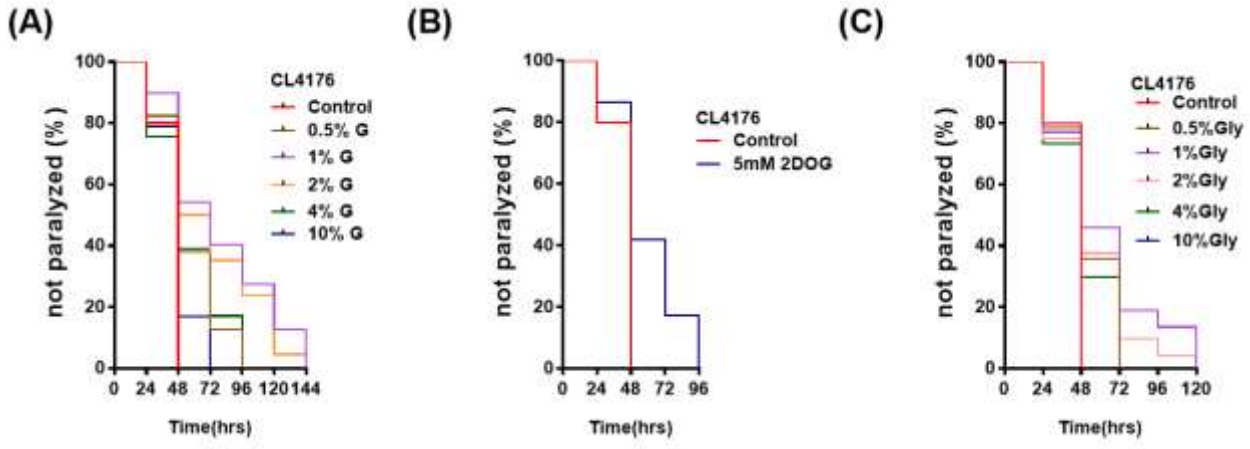

Figure 6.1: Glucose, 2DOG, and glycerol alleviates paralysis due to expression of Aβ in muscles. Standard NGM plates seeded with *E. coli* OP50 were prepared containing different concentrations of glucose or glycerol, or 5mM 2DOG. Worms were assessed for paralysis at 24 hrs interval after temperature upshift. Three independent trials were run for each group, and results were presented as the average. Log-rank test was used to determine any significant change in paralysis behavior between each group. N=50-60 worms per replicate for each data point. (A) Effect of increasing glucose on paralysis of CL4176 strain. (B) Effect of 5mM 2DOG on paralysis. (C) Effect of increasing glycerol on Aβ-mediated paralysis.

Production and accumulation of Aβ in the AD brain is an important hallmark in disease progression. Whereas, accretion of Aβ in human muscles resulted in reduced functioning [513, 514]. Temperature inducible expression of muscle Aβ in *C. elegans* resulted in a time-dependent paralysis phenotype. [515]. Our results showed that supplementation of the growth medium with glucose increases intracellular glucose levels (Figure S6.1) and delays paralysis in a dose-dependent manner. The untreated worms that express Aβ were completely paralyzed within 48 hours with a median time of 24 h. Though addition of 0.5% glucose significantly delayed paralysis ($p<0.0001$, median time=48h, max survival time=72h), the maximum delay in paralysis occurred at 1% glucose ($p<0.0001$, median time=72h, max survival time= 144h). Higher concentrations of glucose increasingly reversed this delay (2%glucose: $p<0.0001$, median time=60h, max survival time= 144h; 4%glucose: $p<0.0001$, median time=48h, max survival time= 96h; 10%glucose: $p=0.059$, median time=48h, max survival time= 72h).

To determine whether the decrease in paralysis due to glucose (Figure 6.1A) is glycolysis dependent, we treated the worms with 5mM 2DOG, an enantiomer of D-glucose. D-glucose cannot be phosphorylated by hexokinase and is thus unable to enter the glycolytic pathway, creating a dietary restriction-like state. 5mM 2DOG significantly reduced paralysis in worms that express Aβ (72 hrs vs 96 hrs, $p<0.0001$, median survival= 48h) (Figure 6.1B). Our results showed glycolysis-independent mechanism of glucose-mediated protection against Aβ- proteotoxicity.

Glycerol can also be used as a carbohydrate energy source and contribute to oxidative phosphorylation [516, 517]. Similar findings as of glucose were observed in worms that express Aβ when fed with glycerol. At 0.5%, 1%, and 2% glycerol in NGM medium, decreased paralysis was observed. Maximum relief against paralysis was observed when worms were fed with 1% glycerol (48 hrs vs 120 hrs, $p<0.0001$, median time= 48h). At higher concentration of glycerol, the paralysis rate began to rise (48 hrs vs 72 hrs, $p=0.026$, median time= 48h) with all worms paralyzed at 10% glycerol within 24 hours. (Figure 6.1C).

Glucose impairs cholinergic neurotransmission in muscle of C. elegans

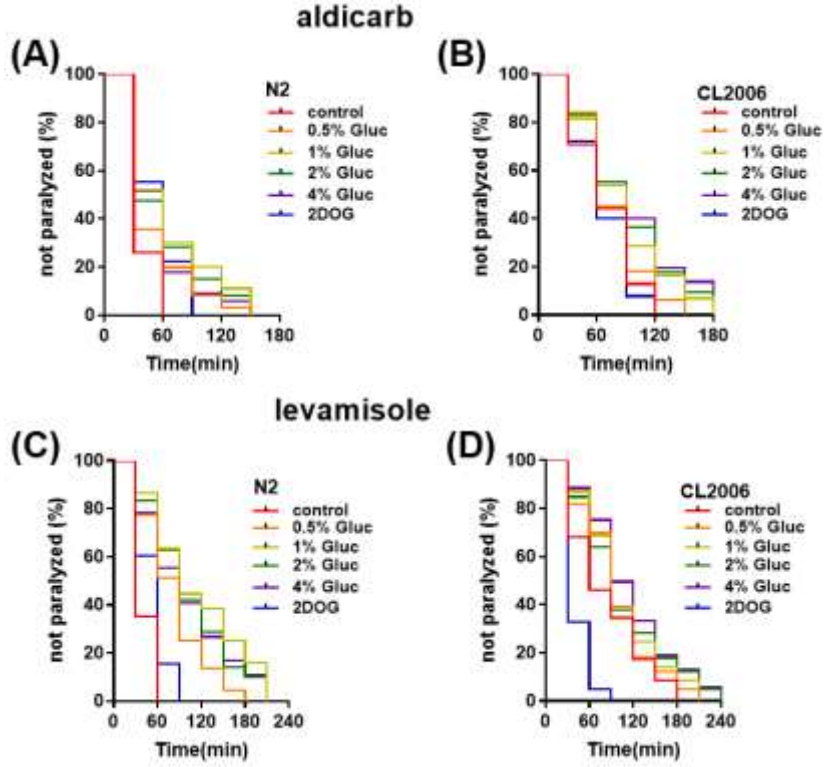

Figure 6.2: Effect of glucose and 2DOG on ACh-mediated neurotransmission. Addition of glucose impaired while 2DOG restores the ACh neurotransmission in muscles of worms that express Aβ. Wild type and Aβ constitutively expressing strain CL2006, were exposed to different concentrations of glucose or 5mM 2DOG for 72h and then transferred to freshly prepared NGM plates containing aldicarb or levamisole. Three independent trials were run for each group, and results were presented as the average. Log-rank test was used to determine any significant change in paralysis behavior between treatments (n = ~50 worms per trial). Table 6.1 shows summary of the statistical analysis. A) Wild type worms in the presence of 1mM aldicarb. B) CL2006 worms in the presence of 1mM aldicarb. C) Wild type worms in the presence of 0.2mM levamisole. D) CL2006 worms in the presence of 0.2mM levamisole.

Expression of Aβ in *C. elegans* muscles is associated with impaired ACh neurotransmission [407]. Given that glucose protects against Aβ-mediated paralysis in *C. elegans*, we were interested to determine whether this improvement was due to improved ACh signaling. Moreover, Aβ expression in *C. elegans* blocks ACh release, any improvement after glucose or 2DOG administration will also effect the Aβ physiological state [57]. The strain CL2006 was used to assess the effect of glucose or 2DOG on pre-existing Aβ pathology while, wild type worms were used as control. The median paralysis time for wild type was 30 min while worms that express Aβ have induced median survival time to 60 min either treated with aldicarb (an ACh esterase inhibitor) or levamisole (a cholinergic agonist). Glucose supplementation of the medium did not restore normal ACh neurotransmission in CL2006, but rather seemed to further impair cholinergic

signaling as it increased resistant to both aldicarb and levamisole. Similar results were observed in wild type worms (Figure 6.2, Table S6.1), indicating that the effect was independent of Aβ.

In the presence of aldicarb, treatment with 5mM 2DOG had no effect on paralysis in Aβ transgenic worms ($p=0.59$), but it increased time to paralysis in wild type worms (60min vs 90 min, $p<0.0001$) (Figure 6.2A and 6.2B). In the presence of levamisole, 5mM 2DOG restored ACh neurotransmission in CL2006 (180min vs 90min, $p<0.0001$) while wild type worms showed resistance against 2DOG (60min vs 90min, $p<0.0001$ (Figure 6.2C and 6.2D).

To evaluate the effect of glucose, glycerol and 2DOG on the Aβ-mediated decrease in fertility, we used strain CL2355 that expresses human Aβ panneuronally along with its matched control CL2122. There was no difference in the number of eggs laid between wild type (N2) and CL2122 worms (294±34 vs 273±23, $p>0.05$), but the time required for the eggs to hatch was delayed in strain CL2122 ($p=0.002$). Expression of Aβ reduced fecundity of CL2355 compared to CL2122 (273±23 vs 157±7, $p<0.001$) and delayed egg hatching with 61% unhatched eggs ($p=0.008$) (Table 2).

Table 6.1: Effect of glucose, glycerol, and 2DOG on worm egg laying and hatching

Treatment	**Total No. of Eggs**	**Unhatched Eggs (%)**			
		Day 0	**Day 1**	**Day 2**	**Day 3**
N2	294±34	100	10.6±1.5	6.1±0.5	3.6±0.8
CL2122	273±23	100	39±7	19.6±3.5	8.6±2.1
CL2355	157±7	100	61.8±4.4	37.5±5.1	17.3±3.4
CL2355 (0.5%G)	164±11	100	40.1±10.9	25.1±3.7	13.1±3.1
CL2355 (1%G)	133±17	100	45.9±3.4	27.7±5.1	17.1±3.9
CL2355 (2%G)	117±8	100	45.5±2.3	27.4±4.5	16.1±2.1
CL2355 (4%G)	109±16	100	58.4±3.1	42.3±5.1	28.5±4.7
CL2355 (10%G)	62±9	100	62.4±1.7	49.4±0.5	40.3±5.1
CL2355 (0.5% GLY)	177±14	100	50.6±6.8	25.9±3.9	14.6±3.6
CL2355 (1% GLY)	214±31	100	44.3±1.3	22.5±4.8	12.1±1.7
CL2355 (2% GLY)	131±12	100	28.1±5.1	16.5±4.1	8.5±3.3
CL2355 (4% GLY)	81±20	100	52.3±8.8	38.5±3.2	30.5±4.6
CL2355 (2DOG)	173±35	100	61.9±3.3	32.6±2.5	19.5±4.2

Transgenic worms expressing Aβ in neurons were synchronized and placed on NGM plates seeded with *E. coli* OP50 containing different concentrations of glucose or glycerol or 5mM 2DOG at 20°C. Adult worms (n=10) were shifted to the fresh NGM plates containing same concentrations of glucose or glycerol or 2DOG and assessed for egg laying and hatching. Adults were removed after 24hrs. We also observed the egg laying and hatching profile in wild type worms fed with only OP50. Total no. of eggs was counted by adding unhatched

eggs and larvae present on plates. Results are presented as Mean ± S.D from three independent experiments.

Supplementation of the growth medium with glucose resulted in a dose dependent decrease in fecundity at concentrations ranging from 1% to 10% in worms that express Aβ (Table 2). Interestingly, we observed a significant increase in fecundity at 1% glycerol (157±7 vs 214±31, $p<0.05$) in worms that express Aβ. From 2% to 4% glycerol however, there was a dose dependent decrease in fecundity. The apparent increase in the number of eggs laid in response to 2DOG was not statistically significant (157±7 vs 173±31, p= 0.432). Glucose, glycerol and 5mM 2DOG each significantly reduced egg hatchability. Worms fed on >2% glucose or glycerol were most severely affected with more than 30% of eggs remaining unhatched by day 3.

Glucose and glycerol, but not 2DOG, induce Aβ oligomerization

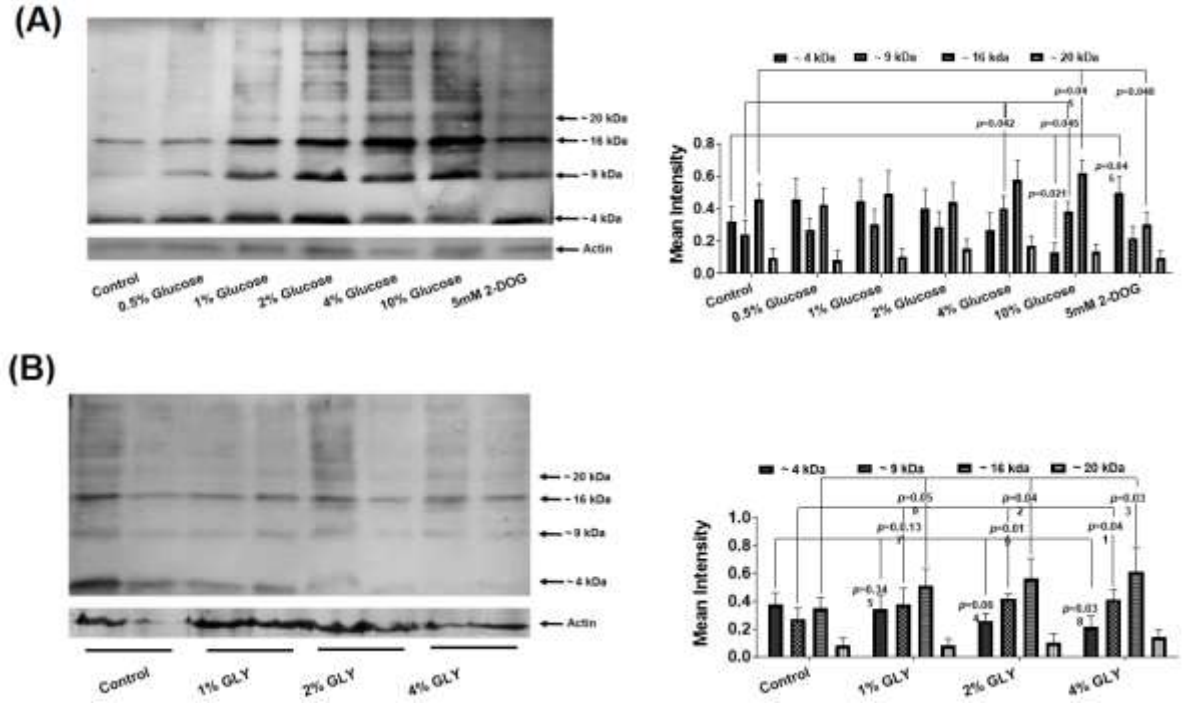

Figure 6.3: Effect of glucose, 2DOG, and glycerol on Aβ oligomerization. Synchronized L1 stage CL4176 worms were treated with v various concentrations of glucose or glycerol, or 5mM DOG at 16ºC for 36 h, followed by 36 h at 23ºC to induce Aβ transgene expression. Total cell lysate proteins were subjected to 16% Tris-Tricine SDS-PAGE and detected by anti-Aβ antibody 6E10. Anti-actin antibody was used as reference control. Original uncropped images with protein ladder (ab116029) are provided in supplemental section (Figure S2). Quantification of Aβ monomers and oligomers appeared on gel was performed using GelQuantNET software. Graphs show results from two biologically independent experiments. A) Aβ transgenic worms were fed with different concentrations of glucose (0.5%-10%) or 5mM 2DOG. B) Western blot of Aβ strain CL4176 grown on medium supplemented with various concentrations of glycerol (1%-4%). Error bars = mean ±SD

As our results seemed to indicate that the deleterious effects of glucose were independent of Aβ, we wished to determine whether glucose had any effect on Aβ oligomerization. The production and accumulation of Aβ oligomers are critical to the progression of AD resulting in long-term potentiation and memory impairments [18, 20, 518]. To investigate Aβ oligomerization, soluble proteins from whole cell lysates of worms grown on glucose, 2DOG, or glycerol were subjected to western blotting. Our results show that higher concentrations of glucose (4% and 10%) decreased the ~4kDa Aβ monomers (1.19 and 2.45-fold, respectively) and substantially increased the ~9kDa oligomers (1.66 and 1.59 fold, respectively) and the ~16kDa oligomers (1.26 and 1.35 fold, respectively) compared to the levels in control worms cultured without glucose. We observed similar results to glucose when worms were fed with different concentrations of glycerol, where a gradual increase in glycerol induced Aβ oligomerization (Figure 6.3B). Interestingly, 5 mM 2DOG was not found to induce Aβ oligomerization. Rather, it significantly increased ~4kDa monomers (1.56 fold, p = 0.045) with a reduction in ~16 kDa oligomers (1.51 fold, p = 0.048) when compared to untreated control (Figure 6.3A).

Glucose induces tau phosphorylation

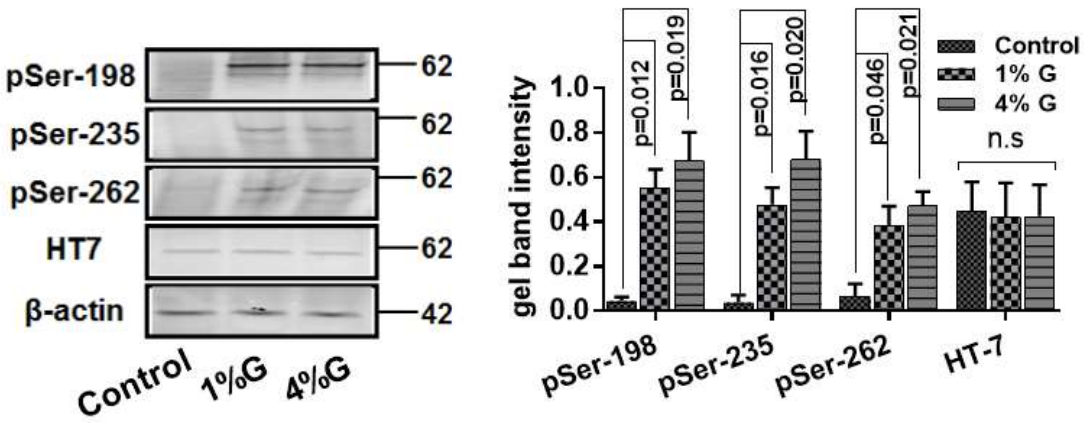

Figure 6.4: Effect of glucose on site-specific phosphorylation of tau expressed in *C. elegans*. Synchronized L1 stage worms of strain VH255 expressing human fetal tau were treated with 1% or 4% glucose for 72 h at 20ºC. Total cell lysate protein was extracted and subjected to western blot. Tau phosphorylation was monitored using different phosphor-tau-antibodies. Total tau was detected using HT7 antibody while, anti-actin antibody was used as reference control. Graphs indicate results from two independent biological trials. Bars= Mean ± S.D

Like Aβ oligomerization, tau hyperphosphorylation is an important contributor to AD pathology as it promotes the formation of neurofibrillary tangles [519]. We examined tau phosphorylation in worms grown on 1% or 4% glucose. We observed increased phosphorylation at tau Ser-198 (1%G, 12.9 fold; 4%G, 15.7 fold), Ser-235 (1%G, 12.8 fold; 4%G, 18.4 fold), and Ser-

262 (1%G, 5.7 fold; 4%G, 7.1 fold) when compared to control worms grown on medium without glucose supplementation (Figure 6.4). No change in the overall level of phosphorylation of the tau protein in response to glucose was observed using the HT-7 antibody. This highlights the specificity of the dramatic changes that were observed using the other antibodies.

Ot-1 suppression modulates Aβ-mediated paralysis in worms that express Aβ in muscle, and tau phosphorylation in human tau-expressing worms.

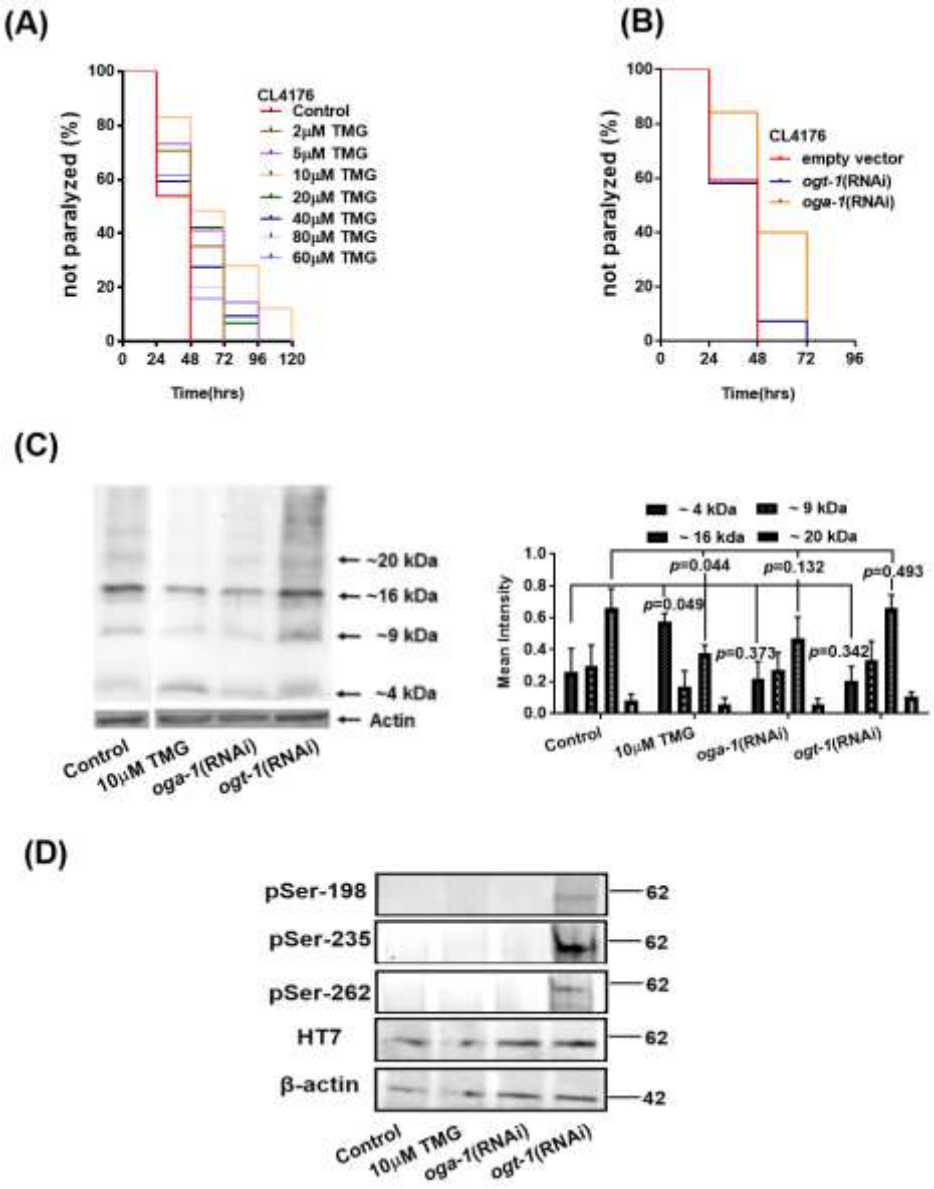

Figure 6.5: Effect of *oga-1* or *ogt-1* suppression on Aβ-mediated paralysis and tau phosphorylation. *Oga-1* was suppressed either by using Thiamet-G (TMG) or RNAi while *ogt-1* suppression was done using specific RNAi. Temperature inducible Aβ worm strain CL4176 and human fetal tau expressing strain VH255 were used in these experiments. A) paralysis profile of worms that express Aβ fed with different concentrations of TMG ranging from 0-

80µM in standard NGM plates. B) Paralysis profile of worms that express Aβ either fed with *ogt-1* or *oga-1* RNAi. Three independent trials were run for each group (n=50-60 worms per trial), and results were presented as the average. Log-rank test was used to determine any significant change in paralysis behavior between each group. C) Effect of 10µM TMG and *oga-1*, and *ogt-1* suppression on Aβ oligomerization in *C. elegans*. D) Effect of 10µM TMG and *oga-1* or *ogt-1* RNAi suppression on site-specific phosphorylation in tau expressing *C. elegans*. After synchronization, VH255 worms were placed at NGM plates containing 10µM TMG or specific *oga-1* or *ogt-1* RNAi. Total cell lysate protein was extracted from 3 days old worms. Tau phosphorylation was monitored using different phosphor-tau-antibodies while total tau protein expression was measured using HT7 antibody. Anti-actin antibody was used as reference control.

Hyperglycemia has been proposed to induce O-β-GlcNAcylation, which in turn has been proposed to impede phosphorylation of adjacent phosphorylation sites [229]. Contrary to expectation, we observed increased phosphorylation of tau in response to increased levels of glucose in the growth medium. To understand the basis of this result, we sought to modulate the level of O-β-GlcNAcylation directly and look at its impact on tau phosphorylation. We used a mutation in the O-GlcNAc transferase gene (*ogt-1*) to prevent O-glycosylation. We also used a mutation in the O-GlcNAcase gene (*oga-1*), as well as thiamet G, an O-GlcNAcase inhibiting drug proposed to treat AD, as both of these prevent O-GlcNAc removal [229, 520-524].

TMG caused a dose dependent delay in paralysis in Aβ transgenic worms (Figure 5A) with the maximum delay, from 24 hours to 72 hours, occurring at 10µM TMG ($p<0.0001$). 10µM TMG was then used in subsequent experiments. Suppression of *oga-1* by RNAi also delayed Aβ-mediated paralysis from 24 hours to 48 hours ($p<0.0001$). In contrast, no change in the median time to paralysis occurred following *ogt-1* gene suppression using RNAi (Figure 6.5B).

The addition of 10µM TMG to the growth medium significantly reduced the levels of Aβ oligomers (~16kDa, 1.75-fold) with a concomitant increase in Aβ monomers (~4kDa, 2.20-fold) compared to untreated control worms (Figure 6.5C). RNAi mediated suppression of *oga-1* or *ogt-1* gene does not affect the Aβ oligomerization pattern in worms. These results show that suppression of *oga-1* by TMG has a different mechanistic effect than RNAi suppression.

We also determined whether O-β-GlcNAcylation can alter tau phosphorylation by modulating O-β-GlcNAcylation levels. In 3 day old worms, suppression of OGA-1 activity, either by TMG or *oga-1* RNAi, has no effect on tau phosphorylation (Figure 6.5D). However, *ogt-1* suppression by RNAi significantly increased phosphorylation on Ser198, Ser235 and Ser262 without affecting total tau (HT7). We found no significant change in tau phosphorylation in control worms. Our results indicate that decreasing O-GlcNAcylation resulted in increased tau phosphorylation. At 7 days of age, tau was phosphorylated in control worms, but this was

significantly reduced by suppression of the *oga-1* gene with RNAi or suppression of the enzyme activity with TMG (Figure S3).

Glucose induces Aβ oligomerization and tau phosphorylation in the presence of TMG

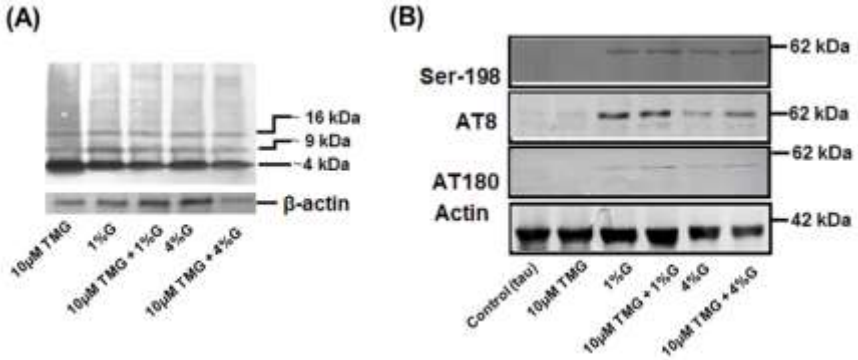

Figure 6.6: Effect of glucose on Aβ oligomerization, tau phosphorylation in the presence of 10μM TMG. Synchronized L1 worms expressing either Aβ (CL4176) or tau (VH255) were fed on standard NGM plates containing no glucose or TMG, with 10μM TMG, or 1% glucose or 4% glucose or a combination of 10μM TMG with 1% glucose or 10μM TMG with 4% glucose. Protein was extracted after 72 hrs. A) Effect on Aβ oligomerization. B) Tau phosphorylation was detected on different epitopes from 3 days old worms using conventional western blot.

Given that glucose increases and TMG decreases Aβ oligomerization, we next investigated the effect of co-treatment. It is evident from Figure 6.6A that in worms that express Aβ, exposure to glucose, either with or without TMG has no effect on Aβ oligomerization. These results show that the amount of TMG used in the current experiment had no protective effect in the presence of elevated glucose.

In worms that express tau, only slight phosphorylation of the tau epitopes critical in AD, AT8 and AT180, was observed in the absence of glucose. The level of phosphorylation was not affected by the presence of TMG (AT8, p=0.461; AT180, p=0.251). Glucose alone or in the presence of TMG considerably increased phosphorylation at AT8 (1% G, 7.50 fold, p=0.024; 1%G + TMG, 10.82 fold, p=0.014; 4% G, 10.01 fold, p=0.014; 4%G + TMG, 10.37 fold, p=0.017, respectively) and AT180 1% G (4.81 fold, p=0.043; 1%G + TMG, 5.77 fold, p=0.032; 4% G, 6.56 fold, p=0.021; 4%G + TMG, 6.95 fold, p=0.017, respectively) (Figure 6B). As with Aβ oligomerization, glucose cause significant phosphorylation of tau and TMG had no protective effect.

TMG increases global O-β-GlcNAcylation, but glucose does not

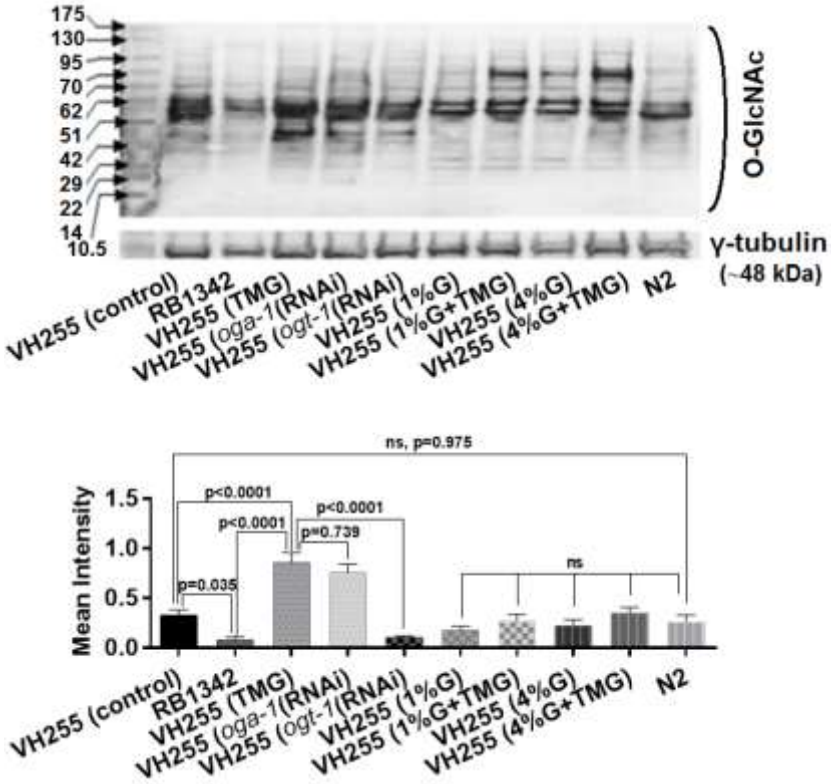

Figure 6.7: Effect of TMG and glucose on global O-β-GlcNAcylation levels in the worms. Synchronized L1 stage worms were fed on standard NGM plates containing no glucose or TMG, with 10µM TMG, or 1% glucose or 4% glucose or a combination of 10µM TMG with 1% glucose or 10µM TMG with 4% glucose. We also examine the effect of *oga-1* and *ogt-1* suppression by RNAi on O-β-GlcNAcylation. Tau transgenic worm strain VH255 fed with OP50 was presented as the tau control. RB1342 (*ogt-1* deletion) and wild type worms (N2) were run parallel as *ogt-1* –ve control and tau –ve control, respectively. Gel band intensities were quantified using GelQuant.NET. The graphs represent results from two independent trials. Error bars = mean ±SD. ~

The ability of glucose to induce phosphorylation of tau contradicts the hypothesis that glucose should increase the level of O-β-GlcNAcylation, thereby inhibiting protein phosphorylation. To investigate this further, we monitored the effect of glucose with and without TMG on tau phosphorylation, using TMG, or RNAi of the genes *oga-1* and *ogt-1* as controls. To check whether TMG and glucose are capable of inducing O-β-GlcNAcylation levels in our study, we fed the tau expressing strain VH255 with 10µM TMG and/ or 1% or 4% glucose. We used an *ogt-1* deletion strain RB1342 and wild type N2 as *ogt-1* –ve and tau –ve controls, respectively. We measured the global change in O-β-GlcNAcylation levels rather than individual protein levels. Our results showed that compared to the transgenic tau control VH255, addition of TMG or suppression of *oga-1* increased O-β-GlcNAcylation levels (0.331±0.052 vs 0.864±0.09, 2.61-fold or 0.331±0.052 vs 0.761±0.08, 2.29-fold), Suppression of the *ogt-1* gene by RNAi or an *ogt-1* deletion mutant reduced overall O-β-GlcNAcylation levels relative to the transgenic control strain

(0.331±0.052 vs 0.086±0.02, 3.93-fold and 0.761±0.08, 3.04-fold). There was no statistical difference between transgenic and wild type controls (0.331±0.052 vs 0.268±0.06, p=0.975), or between the transgenic control and worms fed with 1% glucose (0.331±0.052 vs 0.184±0.03, p=0.086) or 4% glucose (0.331±0.052 vs 0.232±0.05, p=0.207). Furthermore, use of TMG on glucose fed transgenic worms did not influence O-glycosylation relative to the VH255 transgenic control; 1% or 4% glucose in addition of TMG (0.331±0.052 vs 0.0.271±0.06, p=0.431, (0.331±0.052 vs 0.359±0.04, p=0.631). These results clearly showed that TMG or *oga-1* suppression increased overall O-β-GlcNAcylation levels, whereas glucose did not. Exposure to elevated glucose, however, prevented TMG from increasing O-glycosylation.

Discussion

Recent studies have linked lifestyle, i.e. carbohydrate-rich diets, to increased neurodegeneration [525, 526]. In contrast, lower levels of glucose observed in AD post-mortem brains have been interpreted as one of the major factors responsible for disease progression. These seemingly contradictory observations require further studies to characterize the functional role of glucose in neurodegeneration. In this study, we investigated the effect of glucose enrichment on Aβ and tau-mediated toxicity in *C. elegans*. We also explored the role of O-GlcNAcylation in glucose-mediated changes to tau phosphorylation as well as on Aβ pathogenicity by inhibiting expression of genes encoding enzymes for the glycosylation or de-glycosylation of proteins.

It is well documented that the transgenic expression of human Aβ in the body wall muscle cells of *C. elegans* results in progressive paralysis [57, 498]. We found that either 1% glucose or 1% glycerol alleviated paralysis in Aβ transgenic worms. Tauffenberger et al [506] previously found the same result for glucose in worms and suggested that glucose enrichment protects against Aβ proteotoxicity and neurodegeneration. Our results are consistent with this interpretation for both glucose and glycerol. In contrast, in mammalian systems, both glucose and glycerol induce Aβ toxicity [463, 527, 528]. Interestingly, we also found that 2DOG administration significantly reduced Aβ-mediated paralysis, which is consistent with results in mammalian cell lines and mouse models [31, 32]. Understanding this point of contradiction may provide valuable insight into proteotoxicity resulting from hyperglycemia.

We also studied the effect of glucose and 2DOG on ACh neurotransmission in strains that produce Aβ, as extracellular oligomers of Aβ are known to inhibit the release of ACh into the synaptic cleft, resulting in reduced neurotransmission [15, 16, 19]. ACh neurotransmission is essential for muscle contraction, but increased ACh levels can result in paralysis due to muscle overexcitation. As a result, Aβ expression can provide resistance to cholinergic agonists such as

aldicarb, that increases synaptic levels of ACh, or levamisole, that increases ACh receptor sensitization [411, 529]. Aldicarb resistance is used as an indication of pre-synaptic defects whereas resistance against both aldicarb and levamisole is used as an indication of post-synaptic defects [417]. The presence of Aβ inhibits cholinergic signaling both pre- and post-synaptically by creating resistance against ACh release and blocking axonal vesicle clusters [530-532]; we hypothesized that if glucose enrichment can reduce Aβ toxicity, there should be an increase in the sensitivity of Aβ worms to cholinergic hyper-excitation. Contrary to our expectation, glucose induced resistance against both aldicarb and levamisole not only in Aβ-expressing worms but also in wild type. Thus, glucose enrichment is capable of cholinergic inhibition independent of any direct effect it may have on Aβ.

2DOG induced a very modest level of resistance against both aldicarb and levamisole in wild type worms. In vertebrate models, 2DOG administration induced a stress response in neuronal cells that increased resistance against Aβ and other toxins without changing the normal ACh neurotransmission [533, 534]. The partial resistance against aldicarb and levamisole in the presence of 2DOG could be due to the activation of stress resistance proteins as the induced stress response was found to reduces the ACh neurotransmission to compensate for neuronal over-excitation [535]. The major observation, however, is that while 2DOG is unable to increase the toxicity of aldicarb in Aβ-expressing worms, it does restore normal levamisole sensitivity.

Any impairment in ACh signaling could have broad behavioral consequences, e.g. on locomotion, egg laying, mating and muscle contraction [497]. Our observation that glucose does not restore cholinergic neurotransmission related to muscle function indicates that it may likewise fail to restore functions impaired by neuronal expression of Aβ. Machino et al. found lower fecundity and increased egg hatching time in neuronal Aβ expressing worms. [498]. We find that both glucose and glycerol induced further dose-dependent impairment of fecundity and egg viability in worms that express Aβ in neurons. This is also seen in Drosophila and almond moth fed with high glucose or glycerol [536, 537]. Unlike glucose, exposure to 2DOG did not exacerbate the effect of Aβ on fecundity. Although 2DOG was also found to reduce fertility, we observed that 2DOG might be able to compensate the fecundity reduction due to Aβ expression. Overall, our results strongly depict the negative role of glucose in modulating neurotransmission. To help resolve the ambiguous effects of glucose in our study, we examined the effect of glucose enrichment on Aβ oligomerization. Previous studies in mouse models found that hyperglycemia increased Aβ oligomerization, leading to a deterioration of synaptic function [133, 463, 538]. As glucose enrichment was found to induce intracellular glucose levels in *C. elegans* (Figure S6.1, [539]), it is

possible that glucose addition may affect Aβ oligomerization in transgenic worms as well. It has been widely accepted that Aβ monomers are non-toxic, and the accumulation of Aβ oligomers is responsible for Aβ-mediated neurotoxicity as early cognitive decline appears before the Aβ plaque formation in the patients with AD [14-24]. In our study, glucose gradually induced Aβ oligomerization accompanied by decrease in Aβ monomers. These results are in agreement with previous studies where carbohydrate-rich diets accelerated the neurodegeneration by increasing Aβ oligomers [467, 468], and reduction in Aβ oligomerization resulted in improved disease pathology [57, 411, 515]. In our study, we also found that administration of 2DOG reduced disease pathology by decreasing Aβ oligomerization. Similar observation were made in a female mouse model of AD and in cultured neuronal cells, where 2DOG protected against Aβ toxicity by inducing ketogenesis – an alternative energy source of neurons during glucose deprivation [31, 32]. Meanwhile, Aβ oligomers were responsible for both pre- and post-synaptic impairment, induction in Aβ oligomerization explains the failure of restoration to both aldicarb and levamisole sensitivity in our study after glucose enrichment. Our above results indicate that rather being protective, glucose induced Aβ toxicity.

To further elucidate the role of glucose on AD progression, we assessed the effect of glucose enrichment on tau phosphorylation in tau expressing *C. elegans*. We observed induced phosphorylation on critical tau residues Ser198, Ser235, Ser262, AT8 (Ser199/ Ser202/ Thr205) and AT180 (Thr231/ Ser235) after glucose enrichment. The phosphorylation sites examined in our study correlate with severity of neuronal cytopathology in AD leading to conformational changes in tau [507, 519, 540, 541]. Quantitative and kinetic studies have revealed that Ser198/Ser199, Ser202, Thr205, Thr231 and Ser235, are most critical sites in tau pathology and phosphorylation at these sites inhibit tau binding with microtubules, resulting in further phosphorylation of tau and promoting self-aggregation [507]. Increased tau phosphorylation was also found in mouse models of diabetes when they were injected with streptozotocin, which induces insulin dysfunction and hyperglycemia suggesting the negative role of glucose on tau phosphorylation [542-544]. Our results suggest that glucose enrichment in *C. elegans* might result in insulin-resistant like pathology as proposed in past [539].

Modulation of tau phosphorylation could occur due to the induced O-β-GlcNAcylation that negatively regulate phosphorylation. As glucose has been demonstrated to promote the O-β-GlcNAcylation of proteins [226, 229], our results indicate the opposite role of glucose in regulating Aβ oligomerization and tau phosphorylation and we hypothesize that glucose enrichment might not induce O-β-GlcNAcylation. This hypothesis was further strengthened by our observation that

glucose enrichment did not affect the global O-β-GlcNAcylation in our study. Here we proposed that improving O-GlcNAcylation without disturbing normal glucose levels could be beneficial.

For instance, tau expression was found to reduce worm's thrashing rates [58], no improvement in thrashing rates was observed in worms in our study either fed with glucose/ glycerol/ 2DOG or TMG or after suppressing *oga-1* and *ogta-1*(Figure S6.4). These results indicate that reduced thrashing is exclusively associated with tau expression not tau phosphorylation. Similar observations were recorded in the tau expressing *C. elegans* model with pseudohyperphosphorylation, where low or high tau phosphorylation does not affect the thrashing rates [58].

Glucose is an important part of multiple pathways that modulates several cellular mechanisms. In our study, increased glucose concentrations in NGM resulted in induced intracellular levels of glucose in *C. elegans* which is in agreement with an earlier study [539]. Importantly, in our and previous studies using *C. elegans* Aβ models; paralysis assay has been used as a tool to measure Aβ toxicity. The possible reason why glucose enrichment delayed paralysis is the presence of glucose itself in the NGM. Uninterrupted glucose supply can keep the worms alive for extra time even they are continuously experiencing Aβ- toxicity. A recent study by Patridge lab found that increased glucose transport into *Drosophila* neuronal cells by over-expressing glucose transporter GLUT1 reduced Aβ toxicity without reducing overall Aβ protein or mRNA levels. They also observed active flies after GLUT1 induction [545]. Meanwhile, we also found induced worm's speed after glucose administration showing impaired neuronal dysfunction (Figure S6.5). This high glucose administration for longer periods may lead to inhibition of glyoxalase-1 system and production of excess ROS and may enhance disease severity via insulin resistance (47, 48). Our results showed that rather than relying exclusively on paralysis profile, it is important to use other tools to measure Aβ toxicity in *C. elegans*. Collectively, our results suggest that glucose enrichment might not a suitable therapy to cope AD progression; while induction of O-β-GlcNAcylation without increasing the normal glucose level could lead to neuroprotection.

.Supplementary information

Figure S6.1

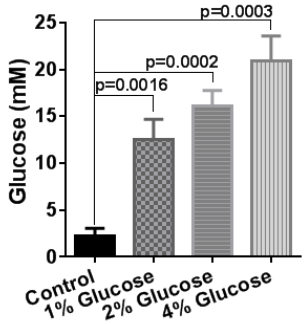

Figure S6.1: Whole-body glucose levels increased after growth on medium supplemented with glucose. Glucose levels in *C. elegans*. Synchronized wild type *C. elegans* were cultured on NGM plates having the concentrations of 0%, 1%, 2%, and 4% glucose. Concentration of glucose was determined at day 3 in whole-body extracts. Final concentrations were normalized by whole-body protein levels and presented as mM. (1% glucose = 55 mM.)

To determine whether the presence of glucose in NGM plates affects intracellular glucose concentrations of *C. elegans*, we cultured *C. elegans* on different percentage concentrations of glucose in NGM media. We found that a gradual increase in external concentration of glucose also induced intracellular levels of glucose in *C. elegans*. At 0% (control), 1%, 2% and 4% of glucose concentration in NGM resulted in intracellular glucose concentration of 2.21±0.82mM, 12.47±2.19mM, 16.09±1.67mM, and 20.86±2.69 mM, respectively. These results showed that increases in external glucose levels also increase intracellular glucose concentration in *C. elegans*.

Figure S6.2

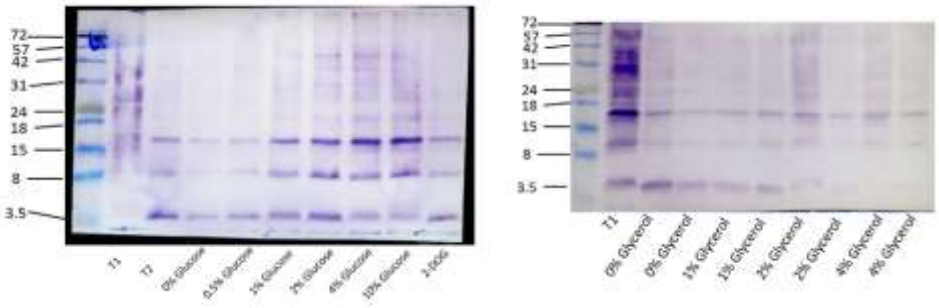

Figure S6.2: Uncropped images of western blots presented in figure 3. Prism ultra-protein ladder (ab116029) was used to estimate protein molecular weights.

Figure S6.3

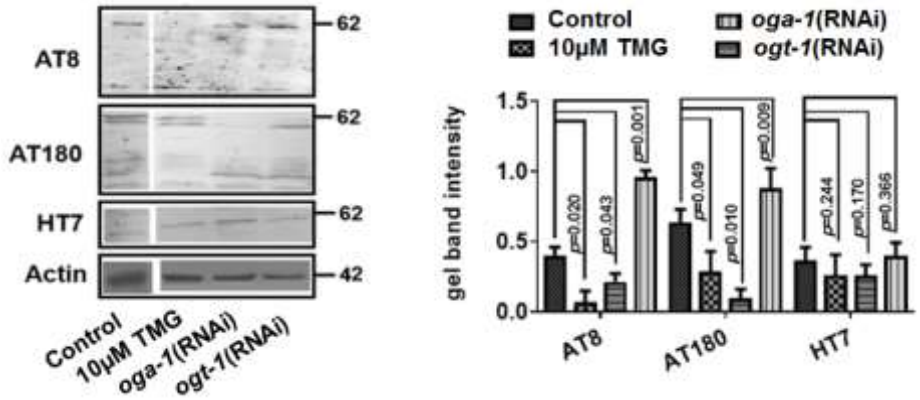

Figure S6.3: Effect of 10μM TMG and *oga-1* or *ogt-1* RNAi suppression on site-specific phosphorylation in tau expressing *C. elegans*. After synchronization, VH255 worms were placed at NGM plates containing 10μM TMG or specific *oga-1* or *ogt-1* RNAi. Total cell lysate protein was extracted from 7 days old worms. Tau phosphorylation was monitored using different phosphor-tau-antibodies while total tau protein expression was measured using HT7 antibody. Anti-actin antibody was used as reference control. The graphs represent results from two independent trials. Error bars = mean ±SD

In 7 day old worms, phosphorylation was apparent in both control and experimental groups. *Oga-1* suppression either by TMG or *oga-1* RNAi knockdown reduced phosphorylation levels at AT8 (5.62 fold, p=0.02; 1.87 fold, p=0.043, respectively) and AT180 (2.21 fold, p=0.049, 6.52 fold, p=0.01, respectively) in 7 days old worms. Meanwhile *ogt-1* knockdown induced phosphorylation at AT8 2.38 fold, p=0.001) and AT180 (1.37 fold, p=0.09) when compared to control. No change in expression of HT7 levels endorses the equal loading of tau protein that was further confirmed by anti-actin antibody. Our results indicate that with *oga-1* suppression, phosphorylation at tau phosphor-enable residues could be modulated.

Figure S6.4:

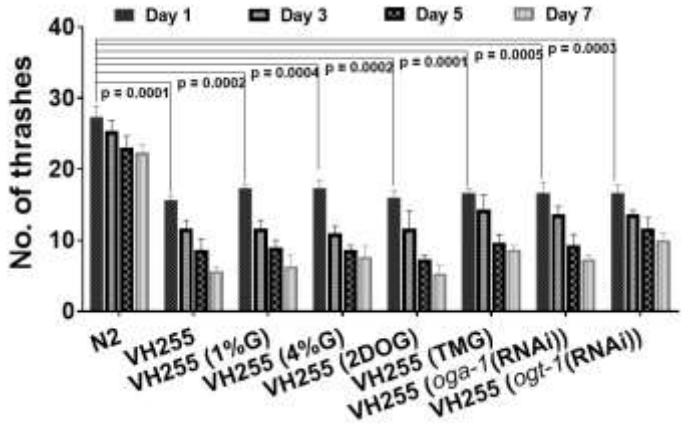

Figure S6.4: Thrashing assay of wild type and tau expressing worms either fed with glucose, 10µM TMG or with *oga-1* and *ogt-1* knockdown. Thrashing rates were counted every 2nd day till day 7. After day 3, worms were shifted to the new NGM plates with 75µM FUDR to block the progeny production. We selected 10 worms randomly from each trial and subjected to count for thrashing in M9 buffer for 10sec at 20°C. Neither treatment improve thrashing rates of tau expressing worms. Results were generated from three independent trials. Error bars = mean ± SD.

Expression of human tau reduces the thrashing rate in transgenic animals [58]. We assessed whether glucose, 2DOG, TMG treatment, and/or *oga-1* or *ogt-1* knockdown affect the trashing rates of tau expressing worms. Tau transgenic worms were assessed for thrashing rates with or without presence of 1% and 4% glucose, or 5mM 2DOG. A gradual decrease in worms thrashing rates was found to be independent of the mode of treatment. We observed similar results for worms fed with TMG or with *oga-1* RNAi or *ogt-1* RNAi.

Figure S6.5:

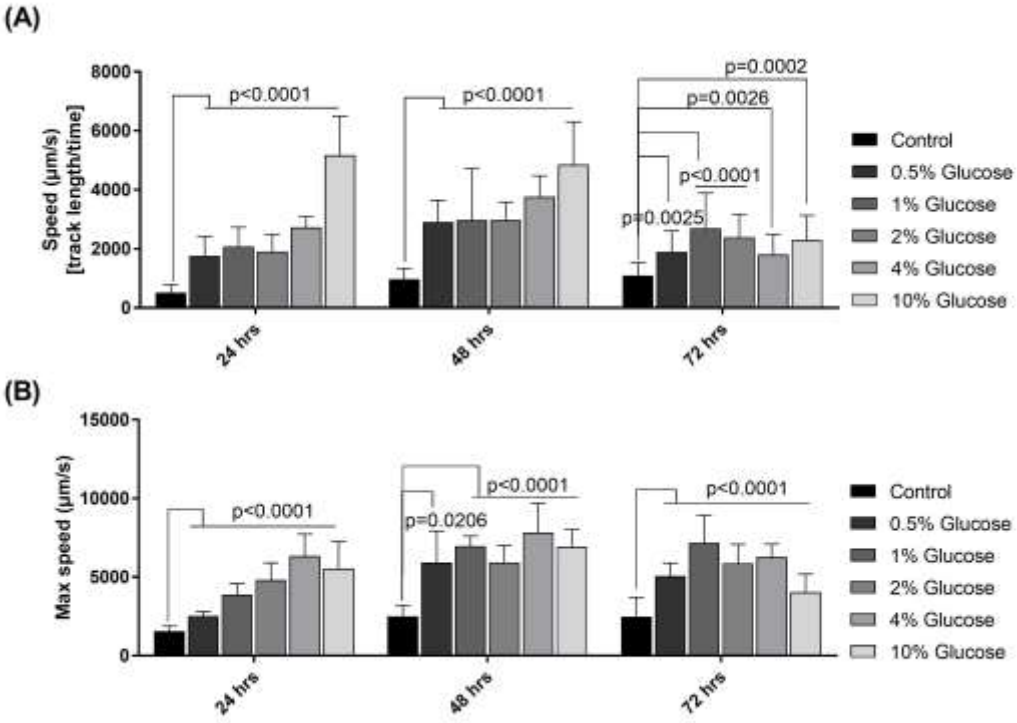

Figure S6.5: Effect of glucose on worm's speed. Synchronized wild type worms were treated with different concentrations of glucose on NGM plates seeded with OP50. Videos (30 sec) were recorded and imported to WormLab 4.0 analytical software after 24 hours. We measured the (A) average speed and (B) maximum speed of the wild type worms fed on glucose. At least 10 worms were selected randomly from each cohort. Error bars = mean±S.D

We observed increased movement of worms that express Aβ in muscle despite induced Aβ oligomerization when treated with glucose. These results were unexpected and we further tested the effect of glucose on movement of the wild type worms. However, after 48 hours, both average and maximum speed starts to drop for worms treated with 4% or 10% glucose suggesting the toxic

effects of glucose at high concentrations. Overall, our results emphasis impaired speed of worms after treatment with glucose.

Table S6.1: Statistical comparison of paralysis curves after exposure to glucose or 2DOG in the presence of aldicarb or levamisole

Treatment	**N2 (aldicarb)**		**N2 (levamisole)**	
	Median time to survival (min)	P value (log rank)	Median time to survival (min)	P value (log rank)
Control	30		30	
0.5% glucose	30	0.0004	90	<0.0001
1%glucose	60	<0.0001	90	<0.0001
2%glucose	60	<0.0001	90	<0.0001
4%glucose	60	<0.0001	90	<0.0001
5mM 2DOG	60	<0.0001	60	<0.0001
Treatment	**CL2006 (aldicarb)**		**CL2006 (levamisole)**	
	Median survival (min)	P value (log rank)	Median survival (min)	P value (log rank)
Control	60		60	
0.5% glucose	60	0.1074	90	0.043
1%glucose	90	0.0011	90	0.009
2%glucose	90	<0.0001	90	0.001
4%glucose	90	<0.0001	90	<0.0001
5mM 2DOG	60	0.589	30	<0.0001

CHAPTER # VII: Metformin attenuates Aβ pathology mediated through levamisole sensitive nicotinic acetylcholine receptors in a *C. elegans* model of Alzheimer's disease

This chapter has been published as" Metformin attenuates Aβ pathology mediated through levamisole sensitive nicotinic acetylcholine receptors in a *C. elegans* model of Alzheimer's disease. Mol Neurobiol. 2016. DOI:10.1007/s12035-016-0085-y "

Abstract

The metabolic disease, type-2 diabetes mellitus (T2DM), is a major risk factor for Alzheimer's disease (AD). This suggests that drugs such as metformin that are used to treat T2DM may also be therapeutic toward AD and indicates an interaction between AD and energy metabolism. In this study, we have investigated the effects of metformin and another T2DM drug, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) in *C. elegans* expressing human $A\beta_{42}$. We found that Aβ expressed in muscle inhibited levamisole sensitive nicotinic acetylcholine receptors and that metformin delayed Aβ-linked paralysis and improved acetylcholine neurotransmission in these animals. Metformin also moderated the effect of neuronal expression of Aβ: decreasing hypersensitivity to serotonin, restoring normal chemotaxis, and improving fecundity. Metformin was unable to overcome the small effect of neuronal Aβ on egg viability. The protective effects of metformin were associated with a decrease in the amount of toxic, oligomeric Aβ. AICAR has a similar protective effect against Aβ toxicity. This work supports the notion that anti-diabetes drugs and metabolic modulators may be effective against AD and that the worm model can be used to identify the specific interactions between Aβ and cellular proteins.

Introduction

Our previous data (Chapter IV-VI of this thesis) showed that glucose enrichment induced Aβ and tau- mediated toxicity in *C. elegans*. While the main cause of AD is still unknown, AD is associated with hyperglycemia and resistance to insulin, indicating that re-sensitizing insulin receptors may be a useful therapy [546, 547]. Insulin resistance leads to type-2 diabetes mellitus (T2DM), an important risk factor in AD development that is associated with impaired insulin signaling and glucose metabolism [480, 481, 548]. A growing body of experimental and clinical research provides evidence that both AD and T2DM share many pathophysiological symptoms, indicating that drugs used for T2DM treatment also may alleviate AD symptoms [1, 49-52].

Metformin is a hypoglycaemic agent that is the primary drug therapy used to treat T2DM. Metformin alters cellular energy metabolism by reducing glucose production by the liver, as well as

by increasing the uptake of glucose into cells. Although the exact mechanism of action of metformin is not understood, metformin increases the NADH/NAD+ ratio and lowers oxygen consumption by blocking Complex I of the mitochondrial electron transport chain [549, 550]. This leads to accumulation of adenosine monophosphate (AMP), resulting in activation of the 5' AMP-activated protein kinase (AMPK) pathway, and thereby, the insulin receptor [551-553].

A limited number of studies have investigated the usefulness of metformin as a treatment for AD and results have been contradictory. Some indicate that metformin may protect against AD by lowering Aβ oligomerization and plaques density. However, work in cell lines shows up-regulation of beta-secretase 1 (BACE1) transcription, which induces Aβ production [386, 554-558]. As a result, it has been suggested that long-term use of metformin may increase the risk of AD in T2DM patients. Taken together, metformin has the potential to influence AD both positively and negatively through multiple mechanisms, though how metformin exerts this influence is poorly understood.

Several vertebrate and invertebrate models are available that express AD-associated human proteins that form aggregates, leading to cellular dysfunction that mimics AD pathology. *C. elegans* is an invertebrate model for which well-established strains are available that express the human Aβ peptide, Aβ [55]. The *C. elegans* model facilitates rapid investigation *in vivo* of the effects of protein aggregation in whole animals, due to a short lifespan and an impressive collection of genetic and epigenetic tools that can be used to investigate the underlying causes of pathology [55, 58]. We are interested in the effect of metabolism on the manifestation of AD. The commonalities between diabetes and AD have led us to investigate metformin in our AD model, as it is the most widely used anti-diabetes drug. A second anti-diabetes drug, Aminoimidazole-4-carboxamide ribonucleotide **(**AICAR) was also tested in this study as it is a potent AMPK pathway activator [559, 560].

Results

Transgenic *C. elegans* strains are available that express human Aβ in muscles or neurons. Expression of the Aβ transgene in muscles of *C. elegans* is associated with severe, age-progressive paralysis and impaired ACh neurotransmission, whereas Aβ expression in *C. elegans* neurons results in behavioral defects and reduced fecundity [55, 405]. The main idea behind this work was to investigate whether treatment with metformin or the AMPK activator AICAR could reverse the adverse effects of Aβ toxicity in *C. elegans*.

Metformin alleviates paralysis caused by expression of human Aβ in C. elegans muscle.

Deposition of Aβ in the AD brain is associated with disease progression, whereas deposition in human muscles in old age results in inclusion-body myositis that impairs proper muscle functioning [405, 514]. In *C. elegans* strain CL4176, expression of Aβ in muscle is temperature inducible, which results in time-dependent deposition of Aβ as well as a time dependent paralysis phenotype [57, 405, 407]. In this study, we found that inclusion of metformin in the growth medium delayed the onset of paralysis in a dose-dependent manner (Figure 7.1).

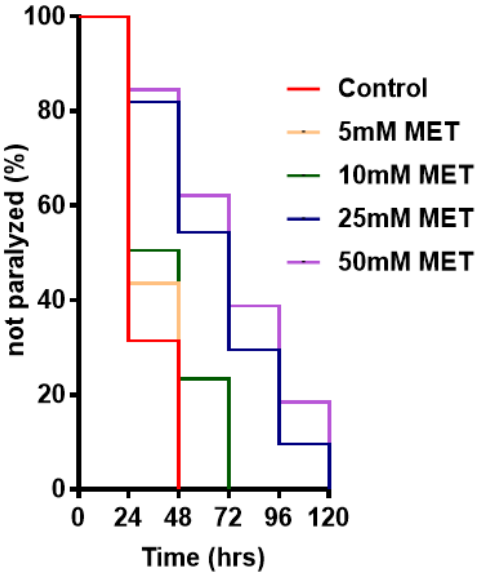

Figure 7.1: Metformin alleviates paralysis due to expression of Aβ in muscle cells. Effect of different concentrations of metformin on Aβ-associated paralysis in the temperature inducible strain, CL4176. Metformin delays paralysis due to Aβ expression in muscle cells. (control vs 25mM metformin, p<0.0001). Time in hours from induction of Aβ expression in strain CL4176 by temperature upshift. A log-rank survival test was applied for comparisons between treatments. Values are percentage of worms that remained active, averaged from three independent trials. n= 40-50 worms per replicate for each data point.

Times to paralysis at different doses of metformin were compared to the untreated control using log-rank survival curves. Median time to paralysis at 0mM metformin was 24 hours. 5mM metformin had no effect on Aβ-mediated paralysis relative to the matched control (p=0.074). Although we observed a significant delay in paralysis at 10mM metformin (p<0.001, median time to paralysis of 48 hours), the maximum delay in paralysis occurred at 25mM (p<0.0001, median time to paralysis of 72 hours). Doubling the dose of metformin to 50mM failed to provide additional protection (p=0.704, median time to paralysis of 72 hours) (Figure 7.1). 25mM metformin was used in subsequent assays.

Metformin improves neurotransmitter function in C. elegans that express human Aβ

Aβ expression in muscle is known to impair ACh neurotransmission in *C. elegans* [407]. As such, the ability of metformin treatment to delay Aβ-mediated paralysis may be the result of improved ACh neurotransmission, an essential element of muscle contraction [561]. Another effect of impaired ACh neurotransmission due to Aβ expression is resistance against ACh -mediated excitotoxicity. Restoration of normal sensitivity to ACh provides an alternative means of monitoring the physiological effect of Aβ. By using strain CL2006 that constitutively expresses Aβ in muscle, we can evaluate the effect of metformin on pre-existing Aβ-mediated pathology [55]. We found that metformin restored normal ACh neurotransmission to CL2006 worms that express Aβ constitutively. This was assessed as restoration of normal sensitivity to aldicarb (an ACh esterase inhibitor) and levamisole (a cholinergic agonist) in the presence of 25mM metformin. As expected, the Aβ expressing worms showed impaired ACh neurotransmission and were therefore less susceptible to excitotoxicity caused by either aldicarb or levamisole than were wild type worms. Exposure to 25mM metformin significantly reduced the resistance of CL2006 toward both aldicarb (120 min vs 180 min, $p<0.0001$) (Figure 7.2A) and levamisole (90 min vs 240 min, $p<0.0001$) (Figure 7.2B). Wild type worms showed slight resistance against aldicarb when treated with metformin ($p=0.032$) but this was not observed for worms that had been treated with levamisole ($p= 0.65$).

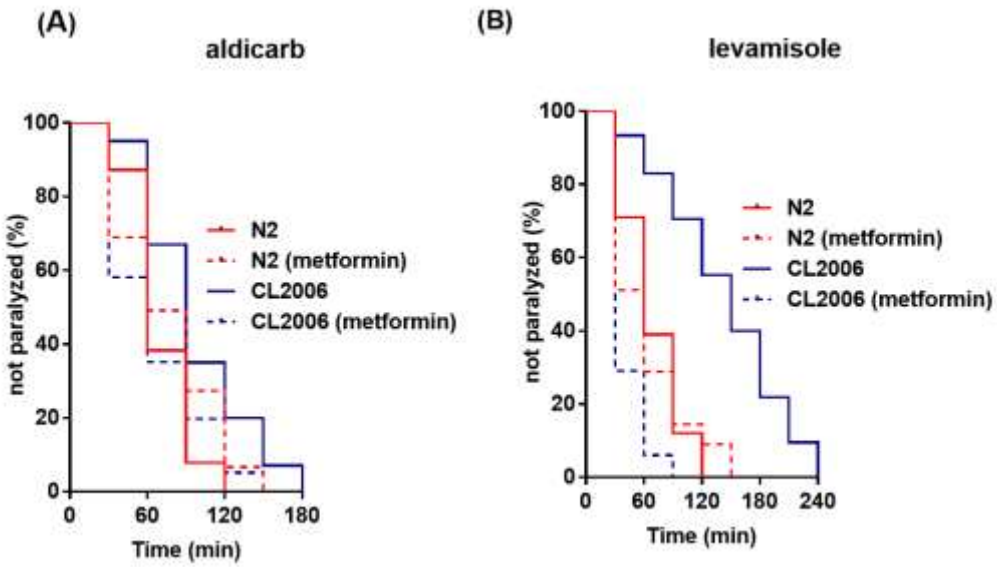

Figure 7.2: Metformin restores ACh neurotransmission in muscle of Aβ-expressing *C. elegans*. Wild type and the strain that expresses Aβ constitutively in muscle, CL2006, were exposed to 25mM metformin for 72 hrs and then transferred to NGM plates containing either aldicarb or levamisole. (A) 25mM metformin plus 1mM aldicarb. (B) 25mM metformin plus 0.2mM levamisole. A log-rank survival test was applied for comparisons between treatments. Values

are percentage of worms that remained active, averaged from three independent trials. n= 40-50 worms per replicate for each data point.

Thus, not only does metformin reduce Aβ-mediated toxicity upon acute induction of Aβ expression, but it can also reverse the ACh neurotransmission dysfunction associated with chronic expression of Aβ. These results encouraged us to evaluate the ability of metformin to alleviate dysfunction of serotonin-mediated behaviour associated with expression of Aβ in nerve cells. Three previously developed bioassays were used to monitor neuronal dysfunction 1) chemotaxis, 2) serotonin sensitivity, and 3) fecundity and egg hatching [57, 498]. For these experiments, we used worms of strain CL2355, in which Aβ is expressed pan-neuronally, together with the matched no-Aβ control strain, CL2122.

Chemotaxis toward benzaldehyde was severely impaired in worms of strain CL2355 following induction of Aβ expression in neurons (0.06±0.02) compared to the no-Aβ control strain, CL2122 (0.22±0.06, p<0.0001) (Figure 7.3A). Exposure to 25mM metformin significantly improved chemotaxis of the Aβ expressing strain which was indistinguishable from the metformin treated control strain (0.176±0.04, p=0.091).

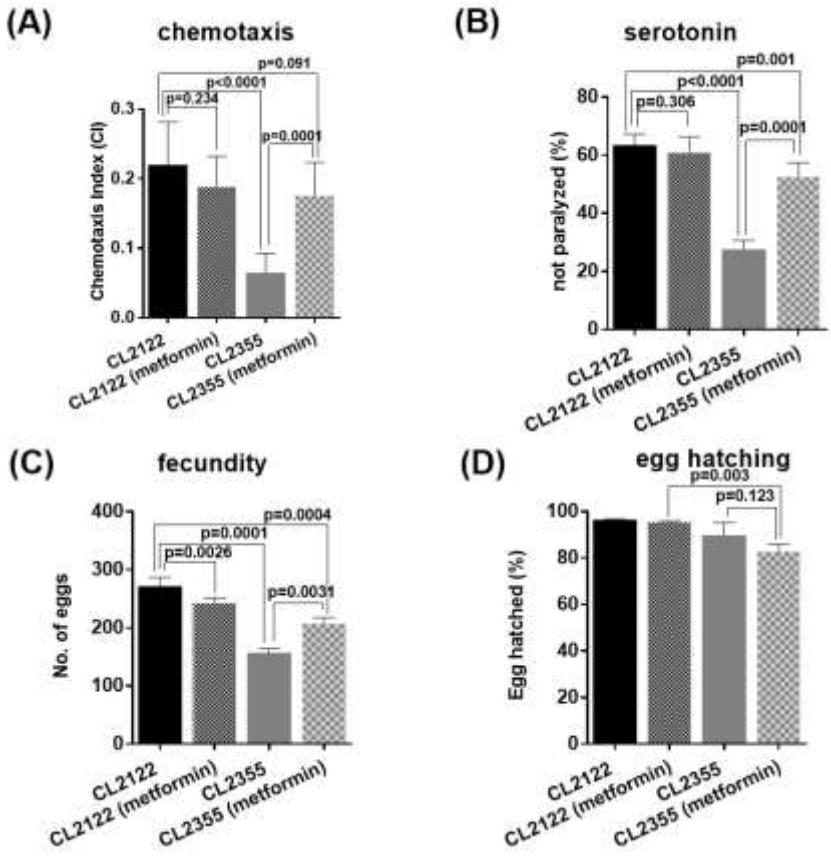

Figure 7.3: Metformin suppresses defects in chemotaxis, serotonin sensitivity, fecundity caused by neuronal expression of Aβ in *C. elegans*. CL2355 is a transgenic strain that

expresses human Aβ in neurons, whereas CL2122 is the matched control strain. Synchronized worms were placed at 16ºC for 36 hrs with or without 25mM metformin and then shifted to a higher temperature prior to testing. (A) Restoration of normal chemotaxis by metformin treatment despite neuronal expression of Aβ. n=50 worms per trial for each treatment. (B) Suppression by metformin of hypersensitivity to 1mM serotonin caused by neuronal expression of Aβ. n=50 worms per trial for each treatment. (C) Improvement in fecundity due to treatment with 25mM metformin despite neuronal expression of Aβ. The progeny of 10 worms were counted per trial. (D) Egg viability was not significantly affected by neuronal expression of Aβ or by exposure to 25mM metformin. Three independent trials were run for all experiments. (Bars = Mean ±SD).

C. elegans becomes paralyzed when exposed to exogenous serotonin. Neuronal expression of Aβ renders worms hypersensitive to serotonin induced paralysis relative to control worms that do not express Aβ [57, 562]. In our experiments (Figure 7.3B) Aβ expression resulted in induced paralysis and only 27.3±3.1% of CL2355 worms remained active compared to 63.3±3.8% activity in the no-Aβ control strain CL2122 ($p<0.0001$). Exposure of the control strain to metformin did not alter serotonin sensitivity, 63.3±3.8 vs 61±5.3% active ($p=0.31$). Strain CL2355, which is hypersensitive to serotonin due to expression of Aβ in neurons, showed an increase in mobility from 27.5±3.1% to 53±4.7% ($p=0.0001$) in response to treatment with 25mM metformin. The level of activity restored to CL2355 by metformin did not differ significantly from that of the treated control strain (53±4.7 vs 61±5.3, $p=0.15$). Our results indicate that metformin is able to reduce hypersensitivity to serotonin caused by neuronal expression of Aβ.

Expression of Aβ in neurons is reported to reduce fecundity [498], which we also observed compared to worms that do not express Aβ, 271.3±17.2 vs 157.0±7.5 ($p=0.0001$). We found that egg laying is significantly improved by treatment with 25mM metformin 157.0±7.5 vs 207±10.5 ($p=0.003$) (Figure 7.3C). While we did casually observe a delay in egg hatching, overall egg viability was not affected by the expression of Aβ (Figure 7.3D). In worms that did not express Aβ, 96.4±0.3% of eggs hatched by day three compared to 89.9±5.4% from the CL2355 strain that expresses Aβ. Treatment of the two strains with metformin resulted in 95.5%±0.5% and 82.6±3.5% viability, respectively. Metformin did not affect egg viability in worms that do not express Aβ ($p=0.06$), nor did it affect viability in the strain that did express Aβ ($p=0.123$).

Metformin protects against Aβ toxicity by reducing Aβ aggregation

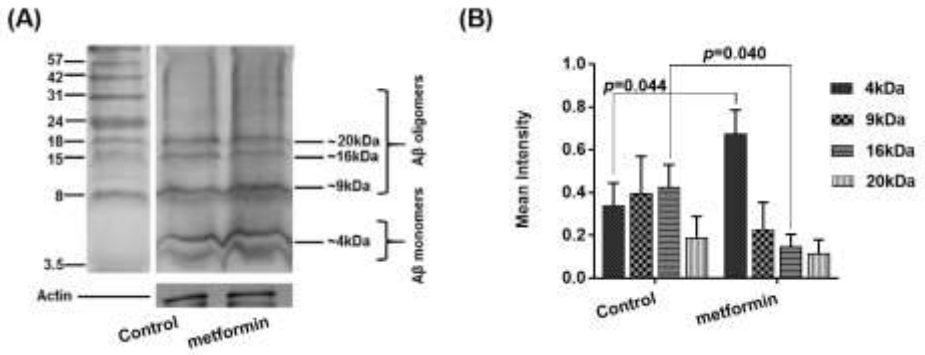

Figure 7.4: Metformin causes a decrease in Aβ oligomerization. Synchronized L1 stage worms of strain CL4176 that contains the temperature inducible Aβ transgene, were treated with 25mM metformin for 36 hrs at 16ºC, followed by 36 hrs at 23ºC to induce transgene expression before protein extraction. Total cell lysate protein was run on a 16% Tris-Tricine gel, blotted and detected with 6E10 antibody to reveal monomers and multimers of Aβ. Anti-actin antibody was used as a reference. Two biologically independent samples were run for each treatment and control (See Figure S7.1). (B) Quantification of Aβ levels relative to actin in each lane was carried out using GelQuantNET software. We observed a significant increase (~1.97 fold) in Aβ monomers (p = 0.044) and a decrease (~2.90 fold) in Aβ oligomers (~16kDa, p = 0.040) due to exposure to 25mM metformin.

Our above observations showed that metformin can reduce Aβ-mediated disruption of both muscular and neuronal function. Although many factors could be involved in reducing Aβ toxicity, a decrease in Aβ oligomerization was found to be protective in previous studies [133, 563]. To determine if the reduction in Aβ-mediated toxicity in metformin-treated, Aβ expressing worms was associated with inhibition in Aβ oligomerization, CL4176 worms were exposed to 25mM metformin after Aβ induction. Protein extracts were subjected to western blotting using anti-Aβ antibody, revealing a decrease in Aβ-oligomers of molecular weight ~16kDa to 0.34x the level in the heat-induced control strain that had not been treated with metformin (p=0.040). A less dramatic, but also significant decrease in ~9kDa (0.58x) and ~20kDa (0.61x) oligomers was observed in transgenic worms treated with 25mM metformin (Figure 7.4A). Along with the decrease in Aβ oligomers, we observed a corresponding 2-fold increase in ~4kDa Aβ monomers in metformin-treated transgenic worms (Figure 7.4B) (p= 0.044).

Metformin causes an increase in Aβ transgene and protein levels in C. elegans

To properly interpret the effect of the observed decrease in oligomerization of Aβ in response to metformin we must know the relative amount of Aβ peptide present. We used quantitative real-time PCR (RT-PCR) to assess Aβ mRNA levels and found that exposure to 25mM metformin for 72 hrs resulted in an ~1.7-fold increase in transcript levels (p=0.038) (Figure 7.5A). To quantify Aβ-peptide levels, we used the anti-Aβ antibody 6E10 on whole cell lysate proteins in a slot blot format. We found an ~2.5-fold increase in total Aβ protein levels (p=0.039) in response to exposure to metformin (Figure 7.5B).

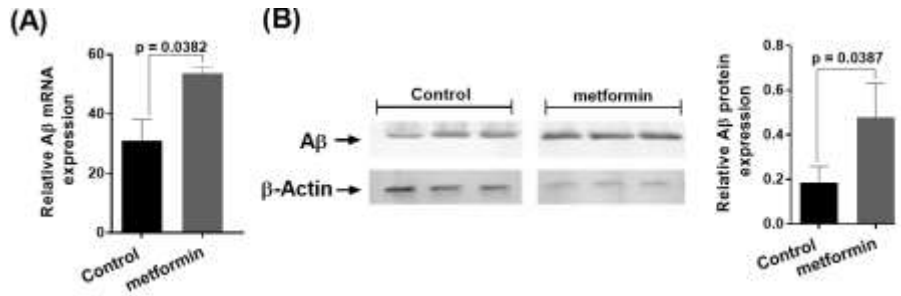

Figure 7.5: Metformin increases Aβ transcript and protein expression in transgenic *C. elegans*. Synchronized L1 stage worms of strain CL4176 that constitutively expresses Aβ in muscle were exposed to 25mM metformin for 72 hrs at 20ºC prior to extraction of total RNA and protein. (A) Results of quantitative PCR from three independent trials showing a significant increase in Aβ mRNA levels. (B) Immunoblot assay of total proteins extracted from the cell lysate. Blots were either incubated with anti-Aβ monoclonal antibody 6E10 for Aβ detection or with anti-actin monoclonal antibody to assess equivalence of protein loading. Aβ immunoreactivity was quantified using GelQuantNET software from non-treated and metformin-treated transgenic worms. After metformin treatment, ~2.5-fold increase in total Aβ protein levels was observed. Error bars = mean ± SD.

AICAR also protects against Aβ-mediated toxicity in C. elegans

Although the mechanism of action of metformin is not fully understood, it does cause activation of the 5'AMP-activated protein kinase (AMPK) pathway [564], which is reported to reduce Aβ toxicity in AD [565]. This suggests that the protection against Aβ-proteotoxicity that we observe in response to metformin could be mediated through the AMPK-pathway. To test this hypothesis, we used AICAR, an analog of adenosine monophosphate known to activate AMPK [566] that we anticipated would mimic the effect of metformin. All doses of AICAR from 0.25 mM to 10 mM significantly decreased paralysis in worms that expressed Aβ. The protective effect of AICAR was dose dependent with a maximal response at 2 mM AICAR ($p < 0.0001$), though the effect at 5 mM was statistically indistinguishable. The efficacy declines at doses above 5mM (Figure 7.6A). The most effective dose, 2mM AICAR, was used in further experiments.

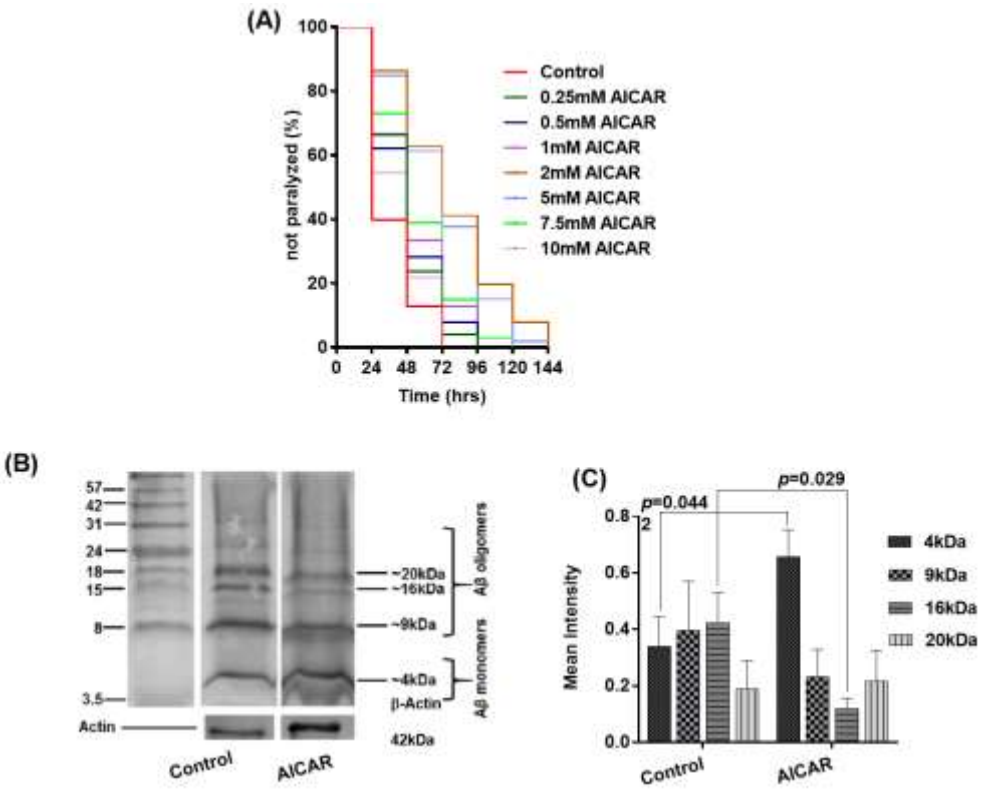

Figure 7.6: Effect of AICAR on Aβ-mediated pathology. (A) Effect of different concentrations of AICAR on Aβ-associated paralysis in the temperature inducible strain, CL4176. AICAR delays paralysis due to Aβ expression in muscle cells. (control vs 2mM AICAR, p<0.0001, paired log-rank survival test). (B) Western blot of Aβ accumulation after 36 hrs upshift of temperature to 23ºC showing the effect of exposure to 2mM AICAR. Aβ was detected using anti-Aβ antibody 6E10. Anti-actin antibody was used as a reference. Two biologically independent samples were run for each treatment and control (See Figure S7.1). (C) Quantification of Aβ levels using GelQuantNET software. A significant increase in Aβ monomers (~1.93 fold, p = 0.042) while a decrease in ~16kDa Aβ oligomers (~3.52 fold, p = 0.029) was observed in transgenic worm treated with 2mM AICAR. A decrease in concentrations of 9kDa (~1.70 fold) and 20kDa (~1.14 fold) was also observed. Error bars = mean ± SD.

We then tested whether the protective effect of AICAR against Aβ-induced paralysis occurred through modulation of Aβ oligomerization. 2mM AICAR significantly reduced the levels of Aβ oligomers, which was reflected in an increase in the level of Aβ monomers (Figure 7.6B). Both AICAR and metformin were found to alter Aβ oligomerization to the same extent (Figures 7.4B & 7.6C).

Discussion

A distinctive feature of AD is the formation of Aβ oligomers, leading to insoluble aggregates. While *C. elegans* expressing human Aβ has limitations as a model of AD, it has the distinct advantage of being amenable to pharmacological, physiological, behavioral, genetic and biochemical studies with large numbers of subjects on a very rapid timescale [56, 567-569]. We use

these advantages to study the potential therapeutic effects of metformin and AICAR on Aβ-mediated pathology, using a *C. elegans* model of AD in which Aβ is expressed in muscle cells or neurons.

Metformin, is a widely-used FDA approved drug to treat diabetes that is also being proposed as a possible treatment against AD. This proposal is supported by the fact that both AD and diabetes share many similar pathological pathways and metformin can reduce AD-like neuropathology in mice [388, 570]. Furthermore, metformin was found to increase lifespan and attenuate the symptoms of old age in *C. elegans* [571, 572], which supports the use of metformin to treat diseases of ageing, such as AD.

Our results show that metformin can alleviate symptoms caused by Aβ expression in muscle, with metformin significantly delaying the onset of Aβ-mediated paralysis and improving ACh neurotransmission. These two outcomes are likely related as paralysis can be caused by impaired ACh neurotransmission. To more precisely define the site of blockage of cholinergic signalling by Aβ, we used activators of cholinergic neurotransmission. We found that Aβ expression was a potent inhibitor of postsynaptic cholinergic neurotransmission mediated by the agonist, levamisole. In contrast, Aβ expression had only a mild inhibitory effect on overall cholinergic signaling induced by the ACh esterase inhibitor, aldicarb. Thus, Aβ seems to block only a specific subset of nicotinic cholinergic receptors in *C. elegans,* a situation that has also been noted for humans [573-576]. We illustrate this in a model described in figure 7.7.

Addition of metformin completely restored to the transgenic worms that expressed Aβ, sensitivity to activators of ACh neurotransmission. Metformin was effective whether the cholinergic activation was mediated by an increase in synaptic ACh levels or by activation of levamisole sensitive ACh receptors. Metformin in the absence of Aβ expression had no effect on the paralysis phenotype, indicating that the effects we observed were specifically related to the expression of Aβ.

ACh can affect interneuronal, neuromuscular and neuroendothelial signalling [577, 578] and is associated directly or indirectly with many *C. elegans* behaviors including muscle contraction, paralysis, locomotion, egg laying and mating [497]. The fact that metformin can restore neuronally controlled phenotypes that had been impaired by Aβ, indicates that metformin also may be able to restore other phenotypes reliant on proper neuronal function. Wu et al [57] previously found that expression of Aβ in neurons reduced the chemotaxis index and caused hypersensitivity to serotonin and that this could be reversed by a natural extract from *G. biloba,* EGb 761. Similarly, we found that metformin was able to improve chemotaxis and reduce hypersensitivity to serotonin. Aβ

expression is also known to decrease both the number of eggs laid and their viability [498]. We find that metformin significantly improves fecundity in worms that express Aβ (Figure 7.3). As ACh and serotonin signaling both modulate egg laying behaviour in *C. elegans* [579-584], it is entirely possible that the improvement in fecundity and egg viability by metformin results from improved function of these signaling systems. The metformin mediated increase in fecundity reflects a decrease in Aβ-mediated toxicity in adults, whereas the improved egg viability probably reflects a decrease in Aβ toxicity at the embryonic stage of development.

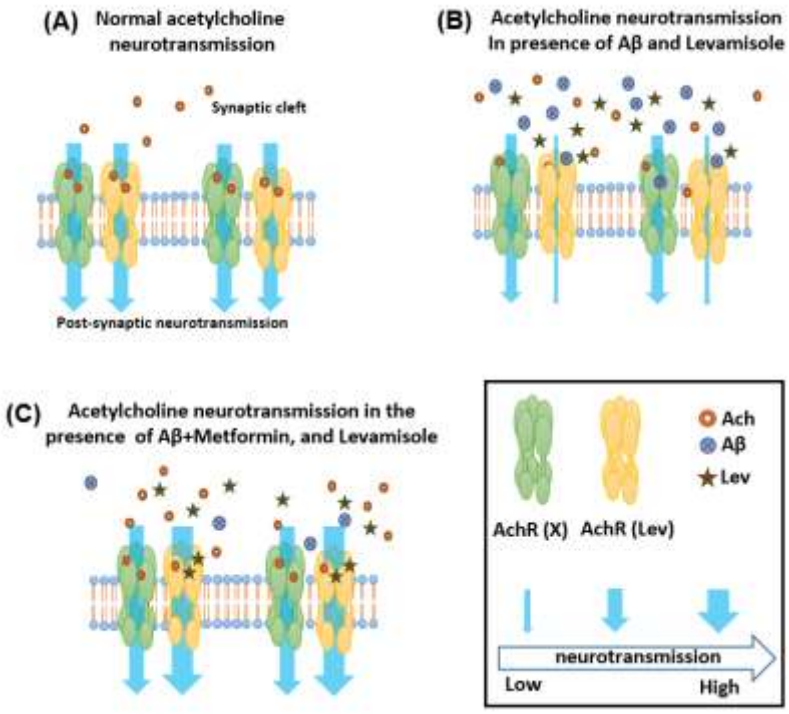

Figure 7.7: Model of the influence of Aβ and metformin on ACh signaling. We found that in *C. elegans*, Aβ inhibits levamisole sensitive ACh receptors. The *C. elegans* levamisole sensitive ACh receptor is a pentamer containing three unique subunits (UNC-29, UNC-38 & UNC-63) that are most homologous to the β2 ACh receptor subunit. In humans, the β2-subunit is found in receptors that bind levamisole and Aβ. These insights have led us to propose the following model. (A) Under normal circumstances, ACh in the synaptic cleft binds to post-synaptic ACh receptors, allowing cations to flow through the central core of any ACh receptors that are present. (B) In the presence of Aβ, the levamisole type receptor is blocked and levamisole is unable to significantly activate its receptor. Other ACh receptors to which Aβ is unable to bind remain active. (C) When metformin is present, the Aβ aggregates decrease and levamisole is strongly activating the ligand gated levamisole receptor. In contrast to levamisole, Aβ has only a small effect on aldicarb-mediated channel activation (not shown) as aldicarb activates all ACh receptors and most are unaffected by Aβ.

We found that metformin had no effect on chemotaxis of worms that did not express Aβ. Metformin did, however, decrease fecundity and egg viability of nematodes that did not express Aβ. As a results, the improvement in fecundity and egg viability caused by the metformin-mediated

decrease in Aβ toxicity did not reach 100% of the control values, probably as a result of the negative effect of metformin itself. Metformin is known to induce some side-effects in patients using metformin therapeutically [585], but it is very widely used despite these limitations.

In order to understand how metformin protects against Aβ-mediated toxicty in *C. elegans*, we evaluated the effect of metformin on Aβ production and oligomerization. Previous studies on vertebrates found that metformin caused a significant increase in Aβ mRNA and protein levels [386, 586]. Because wild type *C. elegans* does not have an APP gene, it does not naturally produce the Aβ peptide. While there is no *a priori* reason why metformin should change expression of the Aβ transgene under the control of the *myo-3* promoter, we did observe a significant induction by metformin of both the Aβ transcript and the corresponding peptide in strain CL2006. Induction in Aβ mRNA in response to a coffee extract was previously observed in worms of strain CL4176 that express Aβ under the control of the *myo-3* promoter [587]. The coffee extracts were also found to be neuro-protective despite the increase in Aβ levels. Although no proper mechanism has been found behind this induction, it suggests that the myosin promoters are responsive to a variety of inducing compounds, at least two of which protect against Aβ. The important point for the current study is that despite the metformin-mediated increase in the level of Aβ peptide, there was still a notable decrease in paralysis and improvement in behavioral phenotypes.

The improved behaviour in the face of increased levels of Aβ is associated with a decrease in the formation of toxic oligomers, as was previously noted in vertebrate systems [21-23, 108]. In AD, aggregates of Aβ oligomers impair synaptic transmission, resulting in damaged long-term potentiation and memory in vertebrate transgenic models [16, 108]. In our study, metformin reduced Aβ oligomerization, resulting in an increased proportion of monomeric peptides (Figure 7.4). The protective effect of metformin was observed despite the CL4176 strain that was used in our study predominantly expressing the $A\beta_{3\text{-}42}$ peptide rather $A\beta_{1\text{-}42}$. $A\beta_{3\text{-}42}$, which constitutes a significant fraction of the Aβ found in the AD brain, self-aggregates more rapidly than $A\beta_{1\text{-}42}$ [412]. In the current study, we found a consistent beneficial effect of metformin on Aβ-mediated toxicity in three different Aβ transgenic strains regardless of the specific length of Aβ peptide known to be expressed in each strain (1-42 or 3-42).

Moreover, Aβ could be found as soluble oligomers or insoluble fibrils in several denaturants like commonly used SDS [588]. In our study, we solubilized protein for extraction in SDS. Metformin reduced Aβ oligomerization of the soluble Aβ oligomers. It is possible that potentially toxic Aβ fibrils remained insoluble in the presence of SDS. Studies on AD have shown that insoluble Aβ plaques do not induce memory impairment unless they are first solubilized to release

Aβ dimers or oligomers, suggesting that the plaques act as reservoirs for soluble Aβ- oligomers rather than being toxic themselves [108, 588, 589]. Taken together our results and those of other studies suggest that minimizing Aβ oligomerization irrespective of overall Aβ levels could be beneficial in AD therapy. Metformin seems to exert its protective influence by inhibiting Aβ oligomerization.

Like metformin, other AMPK activators such as AICAR and resveratrol have also been investigated for their protective properties. AICAR has been shown to reduce Aβ aggregation in neuronal cell lines, whereas resveratrol protects against Aβ-mediated paralysis in worms [590-592]. We demonstrate that not only metformin, but also AICAR, can protect against Aβ-mediated paralysis in worms with a concomitant reduction in Aβ oligomers (Figure 7.6). Role of the AMPK pathway on neuroprotection is poorly understood and a subject of debate. Some studies have found that AMPK activation induced amyloidogenesis, whereas others have found that AMPK activation induced autophagy, resulting in Aβ clearance [593-596]. Moreover, a range of natural products and drugs that up-regulate the AMPK pathway, such as phytic acid, arctigenin and resveratrol, also reduced Aβ-proteotoxicity and improved memory function in mouse models [597]. Therapeutic mechanisms of metformin other than activation of AMPK have been proposed [598], though it has been argued that the doses that have been employed to observe these effect were therapeutically unrealistic [599]. The similarities in our system between the effect of metformin and AICAR suggest that AMPK activation is the mode of action, but this remains to be confirmed. Although the *C. elegans* model expressing human Aβ is limited as a model of AD, it provides a valuable platform for the investigation of potential drugs for protection against Aβ pathology. As metformin has been used as a first-line therapy to treat diabetes, our results support the investigation of diabetic drugs for AD treatment.

Supplementary information
Figure S7.1

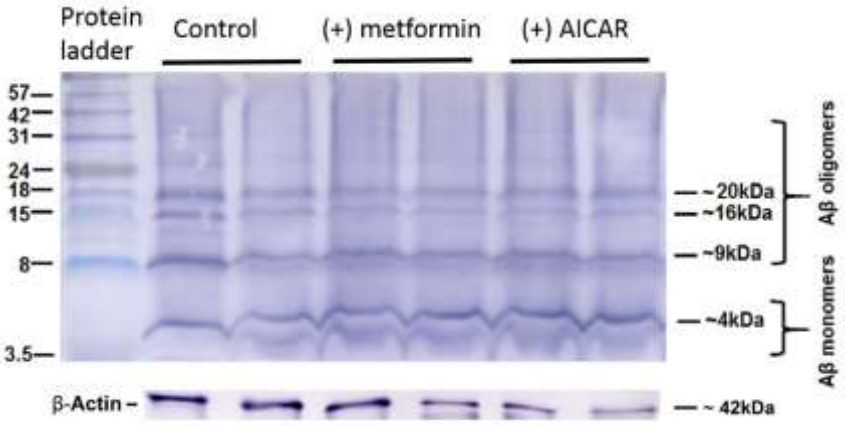

Figure S7.1: Complete figure of immunoblot presented in Figures 7.3 and 7.4

CHAPTER # VIII: General Discussion

AD is a progressive chronic neurodegenerative disorder within the brain without any cure. Pathological hallmarks of AD include production and accumulation of Aβ plaques and NFTs of the tau protein, as well as neuronal loss leading to memory impairment [48]. The exact cause of AD is still enigmatic though several factors are thought to be involved in disease progression [600]. The severity of these pathological markers seems to be associated with impaired glucose metabolism. The exact role of glucose in the progression of AD has not yet been elucidated, due to contradictory observations. The decreased glucose metabolism in AD could be attributed as a main cause [601-603] or protective against AD progression [45] as evidence indicates that impaired mitochondrial bioenergetics may proceed by decades the appearance of disease [426, 604]. Various epidemiological studies showed diabetes (metabolic disease with chronic hyperglycemia) as a major risk factor in AD [164] suggesting that induced glucose may increase neurodegeneration. Meanwhile, improvements in AD symptoms after caloric restriction, or a reduction in glucose dependent energy metabolism supports the hypothesis that a decrease in glucose metabolism is protective [31, 32, 605, 606]. Overall, these observations identify glucose energy metabolism as an important target of study to improve our understanding of AD progression.

To understand how glucose energy metabolism influences the progression of AD, we used a simple model system *C. elegans* that express human Aβ in either muscles or neurons, or *C. elegans* that express human tau protein in neurons. When human Aβ is expressed in *C. elegans* muscles, it results in a progressive paralysis phenotype, while expression in neurons results in impaired behavioral responses. Expression of human tau in *C. elegans* results in reduced mobility (thrashing) and tau phosphorylation. Interestingly, both Aβ and tau expression in worms mimics several key steps of amyloidogenesis and tauopathy underlying gene- and tissue-specific protein misfolding, and toxicity. [53, 55, 58]. When expressed in *C. elegans*, Aβ protein self-aggregates to form fibrillary structures. Tau becomes hyperphosphorylated, just as occurs in AD brains, and conformational changes like hyperphosphorylated tau in humans that resulted in paired helical filaments (PHF).

Genetic association of the core metabolic enzyme DLD with late-onset AD [34]; and reduced activity of DLD-containing enzymes suggests glucose hypometabolism as a key factor in AD [36, 38, 444]. To determine how reduced glucose metabolism affects Aβ or tau toxicity in *C. elegans*, DLD-1 expression was suppressed in transgenic *C. elegans* either by RNAi or by its chemical inhibitor MICA. DLD-1 suppression decreased the formation of toxic Aβ oligomers and restores behavior impaired by Aβ expression. DLD-1 suppression does not change overall Aβ-

mRNA levels, reinforcing the notion that improvement is due to reduction in toxic Aβ oligomers and oxidative stress. The effect of DLD-1 suppression could be reversed using a known DLD activator CaI. Although we could not find a precise mechanism of protection mediated by DLD-1 suppression, we found that it was not mimicked by inhibition of oxidative phosphorylation.

As a part of PDH, DLD-1 suppression could reduce downstream glucose dependent energy metabolism. We wished to directly test the effect glucose metabolism on Aβ and tau toxicity *in vivo*. Transgenic worms were fed with of glucose and glycerol or 2DOG. In a previous study, elevated glucose in NGM delayed the Aβ-mediated paralysis, leading to the proposal that glucose enrichment might be a possible treatment for AD [450], though these authors did not investigate the effect of glucose on Aβ oligomerization or tau phosphorylation. However, in parallel studies on dietary restriction or by using of 2DOG (to decrease glucose dependent energy metabolism) gave the opposite impression that a decrease in glucose metabolism also delay Aβ-mediated paralysis in *C. elegans* [31, 32, 274, 607]. This situation prompted us to further study the role of glucose on Aβ and tau- mediated toxicity. We also found repressed Aβ-associated paralysis in response to either glucose or glycerol enrichment, though very high levels proved to be toxic and induced paralysis. However, impaired ACh neurotransmission and induced Aβ oligomerization after glucose enrichment made scenario complex to draw any conclusions about glucose role in modifying neurodegeneration. It is possible that glucose or glycerol itself acted as a potential food source and kept paralyzed worms responsive until they died due to excessive accumulation of Aβ. In contrast, 2DOG was shown to affect Aβ-oligomerization by reducing toxic Aβ-oligomers, suggesting a possible role of glucose metabolism on neurodegeneration. Resistance of Aβ transgenic worms to ACh agonists and induced Aβ oligomerization in worms after glucose exposure advocates a harmful role of glucose in coping Aβ- toxicity.

To further verify our hypothesis that glucose enrichment induces AD pathogenesis, we tested the effect of glucose on tau phosphorylation. Tau become hyperphosphorylated in AD. This increase in phosphorylation could be minimized either by activation of specific phosphatases or by promoting O-GlcNAcylation of phosphorylated serine (Ser) or threonine (Thr) residues. One hypothesis that reduced glucose energy metabolism decrease O-GlcNAcylation by reducing the excess substrate to the enzyme hexosamine in AD [229] was also tested in our study. It was interesting to see that glucose enrichment significantly induced tau phosphorylation on all the pathological critical phospho-motifs investigated during this study. These results suggest that an increase in glucose in the diet may not reduce phosphorylation by promoting O-GlcNAcylation. We further studied this hypothesis by suppressing the *oga-1* and *ogt-1* genes, normally responsible for

removing or adding the O-GlcNAc molecule (thus enhancing O-GlcNAcylation) on Ser/ Thr residues, respectively. We found that glucose induces tau phosphorylation even in the presence of O-GlcNAcylation enhancers, suggesting that glucose has a negative effect on tau phosphorylation. Although no study has directly observed the effect of O-GlcNAcylation on Aβ-mediated toxicity *in vivo*, here we observed a defensive effect of induced O-GlcNAcylation by TMG in worms expressing human Aβ. Generally, our results suggest protective effect of O-GlcNAcylation on AD in terms of improved behavior and a decrease in Aβ oligomerization and tau phosphorylation. As we found negative effects of glucose enrichment on AD progression, we hypothesized that the anti-diabetic drugs' metformin and AICAR could also be beneficial in AD. Both metformin and AICAR reduced Aβ -mediated toxicity in worms suggesting that anti-diabetic drugs might be helpful to slow/ overcome AD pathogenesis [608].

Conclusions:

Taken together, our study provides a new insight in AD research showing a significant effect of energy metabolism on disease progression. The protective effects of lowering glucose metabolism after DLD-1 suppression/inhibition, or by using 2DOG or metformin indicates a defensive role of reduced glucose energy metabolism in AD progression. Moreover, avoiding high glucose metabolism and inducing O-GlcNAcylation may achieve improved neuro protection. Furthermore, drugs used to cure metabolic disorders should be screened for utility in treating AD.

Appendix

Abstract and poster presented at

3rd Molecular Neurodegeneration Conference, Cannes, France (10-12 Sep, 2013)

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dld-1 suppression attenuates the detrimental effects of amyloid beta deposition in a *Caenorhabditis elegans* model of Alzheimer's Disease

Waqar Ahmad, Paul R Ebert

Amyloid beta (Aβ) aggregation is well studied as a major marker and determinant of Alzheimer's disease (AD) pathology. While less work has been carried out on the role of energy metabolism in AD, there is good evidence that it also contributes to the disease. For example, low metabolic rate and ATP levels are also correlated with AD, which also extends to specific enzymes, metabolites, and proteins associated with glycolysis and the TCA cycle. Dihydrolipoamide dehydrogenase (DLD-1), the subject of this study, is a core metabolic enzyme with specific sequence variants that are associated with increased risk of late onset AD. DLD-1 contributes to four major metabolic multi-enzyme complexes, including *a*-ketoglutarate dehydrogenase (KGDH). A non-DLD subunit of KGDH also has variants that are associated with AD. Additionally, the activity level of the enzyme is significantly inversely correlated with the disease state in humans.

We used a previously published *C. elegans* model of AD that consists of two strains CL2006 and CL4176, which produce human amyloid beta (1-42) in body-wall muscle cells either constitutively or by temperature upshift, respectively. Aβ aggregation results in muscle impairment, observed as paralysis, which is particularly apparent when a muscle stimulant is applied. Using this assay, we chemically or genetically modified the function or abundance of DLD-1, to determine whether this could modify disease progression or remission. We also modified other metabolic functions to see whether the observed effect was specific to DLD.

Expression of human Aβ caused significant paralysis of *C. elegans* in the presence of each of the stimulants, aldicarb and serotonin (5-HT). This effect was completely reversed when the dld-1 gene was suppressed in either the constitutive or the inducible Aβ producing strain. The same effect was observed with chemicals that inhibited metabolic pathways involving DLD-1, but not with an uncoupler of the mitochondrial electron transport chain that depletes ATP production capacity. Thus, it was not energy depletion, per se, that caused the effect. Rather, the critical factor seems to be inhibition of an, as yet loosely defined, DLD-1 containing metabolic pathway. The chemicals that were effective in alleviating the Aβ mediated pathology in the model also decreased Aβ aggregation. Interestingly, one of the chemicals in particular effectively decreased Aβ aggregation and reduced the behavioral pathology regardless of whether the Aβ was pre-expressed or co-expressed with the chemical treatment.

Our results show that inhibition of DLD-1 in our model has protective effects against Aβ toxicity although the precise mechanism is not yet fully understood.

dld-1 suppression attenuates the detrimental effects of amyloid beta deposition in a *Caenorhabditis elegans* model of Alzheimer's Disease

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1. Abstract

We are using the model organism, C. elegans to study the role of energy metabolism in the development of Alzheimer's disease (AD). We use amyloid beta (Aβ) aggregation as a marker of AD pathology and control energy metabolism by modifying the activity of dihydrolipoamide dehydrogenase (DLD). We find that suppression of DLD activity, either genetically or chemically prevents and even reverses symptoms of pathology.

2. Introduction

> Energy metabolism contributes to AD and diabetes is a major risk factor. However, the role of energy metabolism in AD is not well-studied.

> DLD is a key component of energy metabolism as it contributes to four major enzyme complexes that contribute to the TCA cycle, either directly or indirectly. Genetic variants of DLD have been linked to AD, or have variants in a non-DLD subunit of a-ketoglutarate dehydrogenase (KGDH). In addition, the activity level of KGDH is significantly inversely correlated with the disease state in humans.

> C. elegans is an unconventional model for AD research, but has been used extensively to investigate the genetic basis of ageing. The C. elegans $A\beta_{1-42}$ model of AD used in this study expresses the human Aβ peptide in the body wall muscles of the animal and induce paralysis upon peptide aggregation.

3. Strains

Wild type: N2
DLD impaired: *dld-1* (wr4)
Heat inducible Aβ expression: CL2006, CL4176

4. Results

Human peptide $A\beta_{3-42}$ expression in muscle cells of the body wall results in paralysis that can be reversed by *dld-1* suppression.

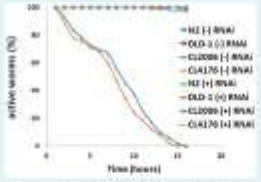

Figure 1. Temperature shift to 25°C induces Aβ peptide aggregation. Strains N2 and DLD-1 produce no human Aβ peptide. Strains CL2006 and CL4176 produce human $A\beta_{1-42}$. (-) RNAi = no epigenetic inhibition of DLD-1. (+) RNAi = epigenetic inhibition of DLD-1.
Key findings: Wild type and *dld-1* mutant worms exhibited normal mobility following heat shift, whereas CL2006 and CL4176 were paralysed within 15 hours, presumably due to Aβ aggregation. Epigenetic suppression of the *dld-1* gene completely prevented paralysis.

Aβ-mediates a decrease in life expectancy that is largely reversed by *dld-1* gene suppression.

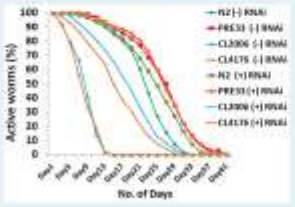

Figure 2. Worms were grown at 23°C to ensure induction and aggregation of human $A\beta_{1-42}$ peptide.
Key findings: The lifespan of the wild type strain (N2) was increased by *dld-1* suppression. The previously published increase in lifespan due to the *dld-1* mutation was observed, but suppression of the *dld-1* gene did not increase lifespan further. Expression and aggregation of the human Aβ peptide reduced the lifespan by half, but most of the decrease was restored by *dld-1* gene suppression.

dld-1 suppression modulates Aβ oligomers and inhibits their deposition in transgenic *C. elegans*

Figure 3 (A): Western blot of Aβ species in the CL2006 and CL4176 *C. elegans* strains. Worms with and without gene suppression by *dld-1* RNAi were upshifted to 23°C to enhance Aβ aggregation. Equal amounts of total protein were loaded in each well. Figure 3 (B): Quantification of gel bands intensities with GelQuant software. (-) RNAi = no epigenetic inhibition of *dld-1*. (+) RNAi = epigenetic inhibition of *dld-1*. C = control strain (N2) without Aβ expression. L= protein size ladder
Key findings: A clear reduction of Aβ oligomers was associated with *dld-1* gene suppression and two immuno-reactive Aβ species at 21 and 28 kDa that are known to be toxic were significantly decreased, whereas the non-toxic Aβ monomers increased (lane 3 and 4).

dld-1 inhibition resulted in lower Aβ deposits in transgenic worms

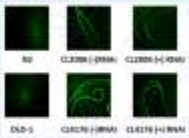

Figure 4. Thioflavin-T has been widely used for detecting misfolded amyloid beta protein. Transgenic worms with and without *dld-1* inhibition were stained with thioflavin-T dye and observed and photographed under Zeiss Axioplan microscope.
Key findings: Aβ deposits were detected in transgenic worms but not in wild type (N2) or *dld-1* mutant strains. A decrease in Aβ deposits due to *dld-1* gene suppression in Aβ expressing worms suggests that *dld-1* inhibition reduces Aβ oligomerization.

dld-1 gene suppression enhanced resistance against aldicarb induced, Aβ-mediated paralysis

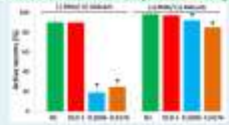

Figure 5. Transgenic worms expressing Aβ were treated with acetylcholine esterase inhibitor, aldicarb at a low dose (0.01mM) with temperature upshift to 23°C and assessed for 24 hrs.
Key findings: Paralysis induced by exposure to low dose aldicarb was prevented by epigenetic suppression of the *dld-1* gene.

dld-1 inhibition relieves paralysis induced by phosphine

Figure 6. Worms were exposed to phosphine at 500 ppm at 23°C. This is the LC_{50} for the N2 strain when exposed for 24 hours. Both the *dld-1* mutation and *dld-1* gene suppression are known to result in resistance against phosphine toxicity.
Key findings: Epigenetic suppression of the *dld-1* gene prevented Aβ-mediated paralysis, indicating that toxicity and induction of paralysis may each be the result of a common mechanism.

Mitochondrial uncoupling induces Aβ-mediated paralysis that is increased by *dld-1* suppression

Figure 7. One effect of *dld-1* suppression is to decrease energy metabolism. To see whether this effect was the mediator of protection against Aβ-mediated paralysis, we exposed worms to a mildly-effective dose of the mitochondrial uncoupler, FCCP (17μM) to limit the capacity to produce ATP.
Key findings: Rather than being protective, exposure to FCCP eliminated the protective effect of *dld-1* gene suppression, suggesting that the protective effect of *dld-1* suppression is not associated with energy depletion.

Neuromuscular excitation results in Aβ-mediated paralysis

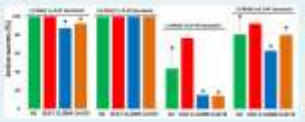

Figure 8. We used serotonin (1mM) exposure to stimulate the body wall muscle cells, a manipulation known to cause paralysis in the worm model.
Key findings: Suppression of the *dld-1* gene significantly relieved paralysis due to serotonin-mediated stimulation. This is consistent with the results in figure 5, when aldicarb was used as a neuromuscular stimulant.

Reduced Aβ-mediated toxicity enhance neurotransmission

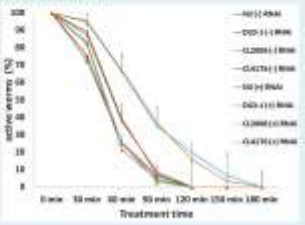

Figure 9. Extended low dose exposure to aldicarb induces Aβ aggregation and paralysis. Aggregated Aβ inhibits neurotransmission and provide short-term protection against excitotoxicity. Worms were exposed to high dosage of aldicarb (1mM), an acetylcholine esterase inhibitor.
Key findings: Exposure to high-dose aldicarb causes excitotoxicity, but because Aβ inhibits neurotransmission it can delay the negative consequences. This protective effect is completely eliminated by suppression of the *dld-1* gene. **Note:** Levamisole, an acetylcholine receptor agonist, gave the same result as treatment with aldicarb.

Chemical inhibition of *dld-1* showed similar effect like epigenetic suppression

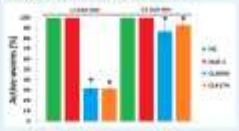

Figure 10. Nematodes were treated with a chemical inhibitor of DLD (DLD-INH) to see if the beneficial effect of *dld-1* gene suppression or mutation could be replicated by drug treatment.
Key findings: Like epigenetic suppression of *dld-1*, chemical inhibition of *dld-1* also showed protective effects against Aβ-mediated paralysis (Fig 10-A). **Note:** Chemical inhibition also reduced the Aβ deposits and oligomers in transgenic worms.

5. Discussion

Inhibition of *dld-1* in Aβ expressing worms either genetically or chemically has protective effects against Aβ-mediated paralysis when it was induced by neuromuscular activation. In contrast, exposure to an uncoupler of the mitochondrial electron transport chain that depletes ATP production capacity strongly exacerbated Aβ-mediated paralysis and eliminated the protective effect of *dld-1* suppression. A chemical inhibitor of DLD has been identified that alleviates Aβ-mediated pathology in the model.

6. Conclusions

Inhibition of *dld-1* in our model has protective effects against Aβ-mediated toxicity although the precise mechanism is not yet fully understood.

7. Acknowledgement

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Abstract submitted to
Genetic Society of Australia Conference, Sydney, Australia (6-9 July, 2014) for oral presentation

Dihydrolipoamide dehydrogenase (DLD) as a potential therapeutic target for Alzheimer's disease

Waqar Ahmad and Paul R Ebert

Altered energy metabolism is associated with Alzheimer's disease (AD) and is proposed to influence disease progression. Amyloid beta (Aβ) plaque formation, a major contributor to AD pathology as well as a marker of disease progression is frequently concurrent with distorted brain metabolism. Unfortunately, in AD pathogenesis, it is still difficult to distinguish cause from consequence. For example, the decrease in energy metabolism associated with AD may be interpreted either as a consequence of, or response to, factors associated with the disease such as elevated levels of oxidative stress. Dihydrolipoamide dehydrogenase (DLD) is a core metabolic enzyme associated with four important mitochondrial enzyme complexes. Interestingly, *dld* gene variants are genetically linked to late-onset Alzheimer's disease (AD); and reduced activity of DLD-containing enzyme complexes has been observed in AD patients. To understand how energy metabolism influences AD progression, we suppressed the *dld-1* gene in worms expressing the human Aβ peptide and also decreased the activity of the DLD enzyme by exposure to the chemical inhibitor, 2-methoxyindole-5-carboxylic acid (MICA). As previously reported, we see that expression of human Aβ in the worm model of AD is associated with decreased lifespan, enhanced paralysis, reduced ACh neurotransmission, hypersensitivity to serotonin, perturbation of chemotaxis and increased Aβ oligomerization. Suppression of either the *dld-1* gene or the activity of its encoded enzyme not only increased lifespan but also alleviated the symptoms associated with expression of human Aβ. Suppression of the *dld-1* gene also results in a decrease in the abundance of toxic Aβ oligomers. These protective effects of *dld-1* suppression seem to be associated with calcium homeostasis.

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